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(54) Title: THERAPY FOR α-GALACTOSIDASE A DEFICIENCY

(57) Abstract

A therapeutic method whereby an individual suspected of having an α-galactosidase A deficiency, such as Fabry disease, is treated either with (1) human cells that have been genetically modified to overexpress and secrete human α-gal A, or (2) purified human α-gal A obtained from cultured, genetically modified human cells.

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## THERAPY FOR α-GALACTOSIDASE A DEFICIENCY
### Background of the Invention

This invention relates to α-galactosidase A and
5 treatment for α-galactosidase A deficiency.

Fabry disease is an X-linked inherited lysosomal
storage disease characterized by symptoms such as severe
renal impairment, angiokeratomas, and cardiovascular
abnormalities, including ventricular enlargement and
10 mitral valve insufficiency. The disease also affects the
peripheral nervous system, causing episodes of agonizing,
burning pain in the extremities. Fabry disease is caused
by a deficiency in the enzyme α-galactosidase A (α-gal
A), which results in a blockage of the catabolism of
15 neutral glycosphingolipids, and accumulation of the
enzyme's substrate, ceramide trihexoside, within cells
and in the bloodstream.

Due to the X-linked inheritance pattern of the
disease, essentially all Fabry disease patients are male.
20 Although a few severely affected female heterozygotes
have been observed, female heterozygotes are generally
either asymptomatic or have relatively mild symptoms
largely limited to a characteristic opacity of the
cornea. An atypical variant of Fabry disease, exhibiting
25 low residual α-gal A activity and either very mild
symptoms or apparently no other symptoms characteristic
of Fabry disease, correlates with left ventricular
hypertrophy and cardiac disease (Nakano et al., New Engl.
J. Med. 333:288-293, 1995). It has been speculated that
30 reduction in α-gal A may be the cause of such cardiac
abnormalities.

The cDNA and gene encoding human α-gal A have been
isolated and sequenced (Bishop et al., Proc. Natl. Acad.
Sci. USA 83:4859, 1986; Kornreich et al., Nuc. Acids Res.
35 17:3301, 1988; Oeltjen et al., Mammalian Genome 6:335-

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338, 1995).  Human α-gal A is expressed as a 429-amino
acid polypeptide, of which the N-terminal 31 amino acids
constitute a signal peptide.  The human enzyme has been
expressed in Chinese Hamster Ovary (CHO) cells (Desnick,
5  U.S. Patent No. 5,356,804; Ioannou et al., J. Cell Biol.
119:1137, 1992); insect cells (Calhoun et al., U.S.
Patent No. 5,179,023); and COS cells (Tsuji et al., Eur.
J. Biochem. 165:275, 1987).  Pilot trials of α-gal A
replacement therapies have been reported, using protein
10  derived from human tissues (Mapes et al., Science 169:987
1970; Brady et al., N. Engl. J. Med. 289:9, 1973; Desnick
et al., Proc. Natl. Acad. Sci. USA 76:5326, 1979), but
there is currently no effective treatment for Fabry
disease.


15                Summary of the Invention
        It has been found that expressing a DNA encoding
human α-gal A in cultured human cells produces a
polypeptide that is glycosylated appropriately, so that
it is not only enzymatically active and capable of acting
20  on the glycosphingolipid substrate which accumulates in
Fabry disease, but is also efficiently internalized by
cells via cell surface receptors which target it exactly
to where it is needed in this disease:  the lysosomal
compartment of affected cells, particularly the
25  endothelial cells lining the patient's blood vessels.
This discovery, which is discussed in more detail below,
means that an individual suspected of having an α-gal A
deficiency such as Fabry disease can be treated either
with (1) human cells that have been genetically modified
30  to overexpress and secrete human α-gal A, or (2) purified
human α-gal A obtained from cultured, genetically
modified human cells.
        Therapy via the first route, i.e., with the
modified cells themselves, involves genetic manipulation

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of human cells (e.g., primary cells, secondary cells, or
immortalized cells) *in vitro* or *ex vivo* to induce them to
express and secrete high levels of human α-gal A,
followed by implantation of the cells into the patient,
5   as generally described in Selden et al., WO 93/09222
(herein incorporated by reference).

When cells are to be genetically modified for the
purposes of treatment of Fabry disease by either gene
therapy or enzyme replacement therapy, a DNA molecule
10  that contains an α-gal A cDNA or genomic DNA sequence may
be contained within an expression construct and
introduced into primary or secondary human cells (e.g.,
fibroblasts, epithelial cells including mammary and
intestinal epithelial cells, endothelial cells, formed
15  elements of the blood including lymphocytes and bone
marrow cells, glial cells, hepatocytes, keratinocytes,
muscle cells, neural cells, or the precursors of these
cell types) by standard methods of transfection
including, but not limited to, liposome-, polybrene-, or
20  DEAE dextran-mediated transfection, electroporation,
calcium phosphate precipitation, microinjection, or
velocity driven microprojectiles ("biolistics").
Alternatively, one could use a system that delivers DNA
by viral vector. Viruses known to be useful for gene
25  transfer include adenoviruses, adeno associated virus,
herpes virus, mumps virus, poliovirus, retroviruses,
Sindbis virus, and vaccinia virus such as canary pox
virus. Although primary or secondary cell cultures are
preferred for the therapy methods of the invention, one
30  can also use immortalized human cells. Examples of
immortalized human cell lines useful in the present
methods include, but are not limited to, Bowes Melanoma
cells (ATCC Accession No. CRL 9607), Daudi cells
(ATCC Accession No. CCL 213), HeLa cells and derivatives
35  of HeLa cells (ATCC Accession Nos. CCL 2, CCL 2.1, and

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CCL 2.2), HL-60 cells (ATCC Accession No. CCL 240),
HT1080 cells (ATCC Accession No. CCL 121), Jurkat cells
(ATCC Accession No. TIB 152), KB carcinoma cells
(ATCC Accession No. CCL 17), K-562 leukemia cells
5  (ATCC Accession No. CCL 243), MCF-7 breast cancer cells
(ATCC Accession No. BTH 22), MOLT-4 cells (ATCC Accession
No. 1582), Namalwa cells (ATCC Accession No. CRL 1432),
Raji cells (ATCC Accession No. CCL 86), RPMI 8226 cells
(ATCC Accession No. CCL 155), U-937 cells (ATCC Accession
10 No. CRL 1593), WI-38VA13 subline 2R4 cells (ATCC
Accession No. CLL 75.1), and 2780AD ovarian carcinoma
cells (Van der Blick et al., Cancer Res. 48:5927-5932,
1988) as well as heterohybridoma cells produced by fusion
of human cells and cells of another species.  Secondary
15 human fibroblast strains, such as WI-38 (ATCC Accession
No. CCL 75) and MRC-5 (ATCC Accession No. CCL 171), may
also be used.

Following the genetic engineering of human cells
with a DNA molecule encoding $\alpha$-gal A (or following
20 another appropriate genetic modification, as described
below) to produce a cell which overexpresses and secretes
$\alpha$-gal A, a clonal cell strain consisting essentially of a
plurality of genetically identical cultured primary human
cells, or, where the cells are immortalized, a clonal
25 cell line consisting essentially of a plurality of
genetically identical immortalized human cells, may be
generated.  Preferably, the cells of the clonal cell
strain or clonal cell line are fibroblasts.

The genetically modified cells can then be
30 prepared and introduced into the patient by appropriate
methods, e.g. as described in Selden et al., WO 93/09222.

Gene therapy in accordance with the invention
possesses a number of advantages over enzyme replacement
therapy with enzyme derived from human or animal tissues.
35 For example, the method of the invention does not depend

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upon the possibly inconsistent availability of sources of
appropriate tissues, and so is a commercially viable
means of treating α-gal A deficiency.  It is relatively
risk-free compared to enzyme-replacement therapy with
5    enzyme derived from human tissues, which may be infected
with known or unknown viruses and other infective agents.
Furthermore, gene therapy in accordance with the
invention possesses a number of advantages over enzyme
replacement therapy in general.  For example, the method
10   of the invention (1) provides the benefits of a long-term
treatment strategy that eliminates the need for daily
injections; (2) eliminates the extreme fluctuations in
serum and tissue concentrations of the therapeutic
protein, which typically accompany conventional
15   pharmacologic delivery; and (3) is likely to be less
expensive than enzyme replacement therapy because
production and purification of the protein for frequent
administration are unnecessary.

As described above, individuals with α-gal A
20   deficiencies may also be treated with purified α-gal A
(i.e. enzyme replacement therapy).  Primary, secondary,
or immortalized human cells genetically modified to
overexpress human α-gal A will also be useful for in
vitro protein production, to produce protein which may be
25   purified for enzyme replacement therapy.  Secondary or
immortalized human cells may be chosen from among those
described above and may be genetically modified by the
transfection or transduction methods also described
above.  After genetic modification, the cells are
30   cultured under conditions permitting overexpression and
secretion of α-gal A.  The protein is isolated from the
cultured cells by collecting the medium in which the
cells are grown, and/or lysing the cells to release their
contents, and then applying standard protein purification
35   techniques.  One such technique involves passing the

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culture medium, or any sample containing human α-gal A,
over a hydrophobic interaction resin such as Butyl
Sepharose® or another resin having a functional moiety
that includes a butyl group.  Passing the sample over
5   such a resin may constitute the first chromatography
step.  If further purification is required, the α-gal A-
containing material eluted from the hydrophobic
interaction resin may be passed over a column containing
a second resin, such as an immobilized heparin resin such
10  as Heparin Sepharose®, hydroxyapatite, an anion exchange
resin such as Q Sepharose®, or a size exclusion resin
such as Superdex® 200.  Preferably, the purification
protocol would include use of each of the above types of
resins.  Alternatively, one could use one or more of the
15  latter resins before or instead of the hydrophobic
interaction resin.

        Previous methods for the preparation of α-gal A
with relatively high purity were dependent on the use of
affinity chromatography, using a combination of lectin
20  affinity chromatography (concanavalin A (Con A)
Sepharose) and affinity chromatography based on binding
of α-gal A to the substrate analog N-6-aminohexanoyl-α-D-
galactosylamine coupled to a Sepharose matrix (Bishop et
al., J. Biol. Chem. 256:1307-1316, 1981).  The use of
25  proteinaceous lectin affinity resins and substrate analog
resins is typically associated with the continuous
leaching of the affinity agent from the solid support
(cf. Marikar et al., Anal. Biochem. 201:306-310, 1992),
resulting in contamination of the purified product with
30  the affinity agent either free in solution or bound to
eluted protein.  Such contaminants make the product
unsuitable for use in pharmaceutical preparations.  Bound
substrate analogs and lectins can also have substantial
negative effects on the enzymatic, functional, and
35  structural properties of proteins.  Furthermore, such

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affinity resins are typically expensive to prepare,
making the use of such resins less suitable for
production on a commercial scale than more conventional
chromatography resins.  Thus, the development of a
5  purification protocol using conventional chromatography
resins, which are readily available in supplies and
quality suitable for large-scale commercial use, is a
significant advantage of the present invention.

An individual who is suspected of having an α-
10  gal A deficiency may be treated by administration of
pharmaceutically acceptable, purified human α-gal A by
any standard method, including but not limited to
intravenous, subcutaneous, or intramuscular injection, or
as a solid implant.  The purified protein may be
15  formulated in a therapeutic composition consisting of an
aqueous solution containing a physiologically acceptable
excipient, e.g. a carrier such as human serum albumin, at
pH 6.5 or below.

The present invention thus provides a means for
20  obtaining large quantities of appropriately glycosylated
and therefore therapeutically useful human α-gal A.  This
makes enzyme replacement therapy for α-gal deficiency
commercially viable, as well as relatively risk-free
compared to therapy with enzyme derived from human or
25  animal tissues.

Skilled artisans will recognize that the human
α-gal A DNA sequence (either cDNA or genomic DNA), or
sequences that differ from it due to either silent codon
changes or to codon changes that produce conservative
30  amino acid substitutions, can be used to genetically
modify cultured human cells so that they will overexpress
and secrete the enzyme.  It is also possible that certain
mutations in the α-gal A DNA sequence will encode
polypeptides that retain or exhibit improved α-gal A
35  enzymatic activity (as would be apparent by expressing

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the mutant DNA molecule in cultured cells, purifying the
encoded polypeptide, and measuring the catalytic
activity, as described herein). For example, one would
expect conservative amino acid substitutions to have
5 little or no effect on the biological activity,
particularly if they represent less than 10% of the total
number of residues in the protein. Conservative
substitutions typically include substitutions within the
following groups: glycine, alanine; valine, isoleucine,
10 leucine; aspartic acid, glutamic acid; asparagine,
glutamine; serine, threonine; lysine, arginine; and
phenylalanine, tyrosine.

Production of α-gal A by the cells can be
maximized by certain genetic manipulations. For example,
15 the DNA molecule that encodes α-gal A may also encode an
heterologous signal peptide, such as the signal peptide
of human growth hormone (hGH), erythropoietin,
Factor VIII, Factor IX, glucagon, the low density
lipoprotein (LDL) receptor, or a lysosomal enzyme other
20 than α-gal A. Preferably, the signal peptide is the hGH
signal peptide (SEQ ID NO:21), and is at the N-terminus
of the encoded protein. The DNA sequence encoding the
signal peptide may contain an intron such as the
first intron of the hGH gene, resulting in a DNA sequence
25 such as SEQ ID NO:27 (see also Fig. 10). Furthermore,
the DNA molecule may also contain a 3' untranslated
sequence (UTS) that is at least 6 nucleotides in length
(in contrast to the α-gal A mRNA found in humans, which
has no 3' UTS, the requisite polyadenylation site being
30 within the coding sequence). The UTS is positioned
immediately 3' to the termination codon of the coding
sequence, and includes a polyadenylation site. It is
preferably at least 6 nucleotides in length, more
preferably at least 12, and most preferably at least 30,
35 and in all cases it contains the sequence AATAAA or a

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related sequence which serves to promote polyadenylation.
A DNA molecule as described, i.e., encoding an hGH signal
peptide linked to α-gal A and containing a 3' UTS that
includes a polyadenylation site, and preferably including
5   expression control sequences, is also within the
invention.  Also within the scope of the invention is a
DNA molecule encoding a protein that includes the signal
peptide of hGH linked to α-gal A or any other
heterologous polypeptide (i.e., any polypeptide other
10  than hGH or an analog of hGH).  The heterologous
polypeptide is typically a mammalian protein, e.g. any
medically desirable human polypeptide.

        Other features and advantages of the invention
will be apparent from the detailed description that
15  follows, and from the claims.

        The term "genetically modified," as used herein in
reference to cells, is meant to encompass cells that
express a particular gene product following introduction
of a DNA molecule encoding the gene product and/or
20  regulatory elements that control expression of a coding
sequence.  The introduction of the DNA molecule may be
accomplished by gene targeting (i.e., introduction of a
DNA molecule to a particular genomic site); furthermore
homologous recombination allows replacement of the
25  defective gene itself (the defective α-gal A gene or a
portion of it could be replaced in a Fabry disease
patient's own cells with the whole gene or a portion
thereof)).

        The term "α-gal A," as used herein, means α-gal A
30  without a signal peptide, i.e., SEQ ID NO:26 (Fig. 9).
There is some indication that residues 371 to 398 or 373
to 398 of SEQ ID NO:26 (Fig. 9) may be removed in the
lysosome; however, removal of this putative propeptide is
not believed to affect activity of the enzyme.  This
35  suggests that any portion of the putative propeptide

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could be deleted without affecting activity.  Thus, the
term "α-gal A" as used herein also covers a protein
having a sequence corresponding to SEQ ID NO:26 except
lacking up to 28 residues at the C-terminus of that
5 sequence.

By "α-gal A deficiency" is meant any deficiency in
the amount or activity of this enzyme in a patient.  The
deficiency may induce severe symptoms as typically
observed in males who are suffering from Fabry disease,
10 or may be only partial and induce relatively mild
symptoms as can be seen in heterozygous female carriers
of the defective gene.

As used herein, the term "primary cell" includes
cells present in a suspension of cells isolated from a
15 vertebrate tissue source (prior to their being plated,
i.e., attached to a tissue culture substrate such as a
dish or flask), cells present in an explant derived from
tissue, both of the previous types of cells plated for
the first time, and cell suspensions derived from these
20 plated cells.

"Secondary cells" refers to cells at all
subsequent steps in culturing.  That is, the first time a
plated primary cell is removed from the culture substrate
and replated (passaged), it is referred to as a secondary
25 cell, as are all cells in subsequent passages.

A "cell strain" consists of secondary cells which
have been passaged one or more times; exhibit a finite
number of mean population doublings in culture; exhibit
the properties of contact-inhibited, anchorage dependent
30 growth (except for cells propagated in suspension
culture); and are not immortalized.

By "immortalized cell" is meant a cell from an
established cell line that exhibits an apparently
unlimited lifespan in culture.

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By "signal peptide" is meant a peptide sequence
that directs a newly synthesized polypeptide to which it
is attached to the endoplasmic reticulum for further
post-translational processing and/or distribution.

5        The term "heterologous signal peptide," as used
herein in the context of α-gal A, means a signal peptide
that is not the human α-gal A signal peptide (i.e., that
encoded by nucleotides 36-128 of SEQ ID NO:18). It
typically is the signal peptide of some mammalian protein
10 other than α-gal A.

The term "first chromatography step" refers to the
first application of a sample to a chromatography column
(all steps associated with the preparation of the sample
are excluded).

15        Brief Description of the Drawings

Fig. 1 is a representation of the 210 bp probe
that was used to isolate α-gal A from a human fibroblast
cDNA library (SEQ ID NO:19). The sequence is from exon 7
of the α-gal A gene. The probe was isolated from human
20 genomic DNA by the polymerase chain reaction (PCR). The
regions underlined in the figure correspond to the
sequences of the amplification primers.

Fig. 2 is a representation of the sequence of the
DNA fragment that completes the 5' end of the α-gal A
25 cDNA clone (SEQ ID NO:20). This fragment was amplified
from human genomic DNA by PCR. The regions underlined
correspond to the sequences of the amplification primers.
The positions of the NcoI and SacII restriction
endonuclease sites, which were used for subcloning as
30 described in Example IA, are also shown.

Fig. 3 is a representation of the sequence of
α-gal A cDNA, including the sequence that encodes the
signal peptide (SEQ ID NO:18).

Fig. 4 is a schematic map of pXAG-16, an α-gal A
35 expression construct that includes the

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CMV (cytomegalovirus) promoter and intron, the hGH signal
peptide coding sequence and first intron, the cDNA for α-
gal A (i.e., lacking the α-gal A signal peptide sequence)
and the hGH 3' UTS.

5          Fig. 5 is a schematic map of pXAG-28, an α-gal A
expression construct that includes the collagen Iα2
promoter, a β-actin intron, the hGH signal peptide coding
sequence and first intron, the cDNA for α-gal A (i.e.,
lacking the α-gal A signal peptide sequence) and the hGH
10  3' UTS.

           Fig. 6 is a chromatogram of the α-gal A
purification step using Butyl Sepharose® resin.  The
absorbance at 280 nm (plain line) and α-gal A activity
(dotted line) of selected fractions is shown.

15         Fig. 7 is a line graph depicting internalization
by Fabry fibroblasts of human α-gal A prepared in
accordance with the invention.  The intracellular α-gal A
activity and total protein concentration were measured
after incubation of the cells with increasing
20  concentrations of human α-gal A prepared in accordance
with the invention.  The effects of the potential
internalization-inhibitors mannose-6 phosphate (M6P; open
diamonds) and mannan (open circles) are shown.

           Fig. 8 is a schematic diagram of the experimental
25  paradigm designed to examine Fabry fibroblasts following
internalization of α-gal A.  The α-gal A activity of the
Fabry cells is measured after exposure to either normal
or α-gal A-overexpressing human fibroblasts cultured in
Transwell" inserts.  "M6P" = mannose-6 phosphate;
30  "Untrf HF" = untransfected human fibroblasts; "BRS11" = a
transfected, α-gal A-overexpressing fibroblast strain.

           Fig. 9 is a representation of the human α-gal A
amino acid sequence (SEQ ID NO:26).

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Fig. 10 is a representation of the DNA sequence encoding the hGH signal peptide and containing the first hGH intron (underlined) (SEQ ID NO:27).

Fig. 11 is a representation of the DNA sequence
5 encoding the hGH signal peptide without the intron (SEQ ID NO:22).

Fig. 12 is a representation of the amino acid sequence of the hGH signal peptide (SEQ ID NO:21).

Fig. 13 is a representation of the cDNA sequence
10 encoding human α-gal A (without signal peptide) (SEQ ID NO:25).


## Detailed Description

Lysosomal enzymes such as α-gal A are targeted to the lysosomal compartment of a cell through interaction
15 with the mannose-6-phosphate (M6P) receptor, which binds to M6P residues present in the oligosaccharide moieties of enzymes destined for the lysosomal compartment (Kornfeld, S. and Mellman, I, Ann. Rev. Cell Biol. 5:483-525, 1989). The primary interaction occurs in the Golgi,
20 where enzymes bound to Golgi M6P receptors are segregated for transport to the lysosomes. A secondary type of interaction is believed to take place between extracellular α-gal A and M6P receptors at the cell surface. Extracellular substances internalized by cells
25 are transported through the cytoplasm in endocytic vesicles, which fuse with primary lysosomes and empty their contents into the lysosomes. In this process, cell surface M6P receptors are also incorporated into endocytic vesicles and transported to lysosomes.
30 Any α-gal A present in the extracellular milieu can, if it bears M6P residues, bind to the cell surface M6P receptors and thereby be transported into the lysosomal compartment along with the receptors. Once in the lysosomal compartment as a result of this scavenger

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pathway, the enzyme can carry out its appropriate
function. Thus, even if a cell is genetically deficient
in producing its own α-gal A, there exists a mechanism
for it to take up exogenously produced enzyme, provided
5    that (a) the enzyme is suitably glycosylated and (b) the
deficient cell bears M6P receptors.

In Fabry disease, vascular endothelial cells of
the kidney and heart have been shown to display severe
histopathologic abnormalities and contribute to the
10   clinical pathology of the disease; these cells, which do
carry M6P receptors, are a particular target of the
presently claimed invention. The α-gal A produced in
accordance with the invention may be delivered either
locally or systemically to the affected cells, by gene
15   therapy (i.e., by genetically modified cells which
express and secrete the glycosylated enzyme within the
patient), or by conventional pharmacologic routes of
administration. An α-gal A in which M6P is present in
the N-linked oligosaccharides is therefore of great
20   importance for therapy in accordance with the invention.

Furthermore, the degree to which the N-linked
oligosaccharides of α-gal A are modified by sialylation
is also of great importance. In the absence of
appropriate sialylation, α-gal A will be rapidly cleared
25   from the circulation due to binding by hepatic
asialoglycoprotein receptors, followed by internalization
and degradation by hepatocytes (Ashwell and Harford, Ann.
Rev. Biochem. 51:531-554, 1982). This decreases the
amount of α-gal A available in the circulation for
30   binding to M6P receptors on cells which contribute to the
clinical pathology of Fabry disease, such as the vascular
endothelial cells of the kidney and heart. Surprisingly,
the Applicants have found that α-gal A secreted by stably
transfected human cells has glycosylation properties
35   which are suitable for the treatment of Fabry disease by

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either gene therapy or by conventional pharmaceutical
administration of the purified secreted protein.  This is
in contrast to the situation with the best-studied
lysosomal enzyme, glucocerebrosidase, in which delivery
5    of the enzyme purified from human placenta or secreted
from transfected CHO cells to the clinically-relevant
cells in the body requires complex enzymatic modification
of the enzyme following purification (cf. Beutler, New
Engl. J. Med. 325:1354-1360, 1991).
10          The therapy of the invention can be carried out in
either of two general ways:  by introducing into the
patient a therapeutically effective amount of purified
human α-gal A obtained from cultured human cells
genetically modified to overexpress and secrete the
15   enzyme, or by introducing the overexpressing cell itself
into the patient.  Techniques to accomplish the necessary
genetic modifications are discussed below, as are the
methods of purification, formulation, and treatment.


Example I.  Preparation and Use of Constructs Designed
20   to Deliver and Express α-Gal A
          Two expression plasmids, pXAG-16 and pXAG-28, were
constructed.  These plasmids contain human α-gal A cDNA
encoding the 398 amino acids of the α-gal A enzyme
(without the α-gal A signal peptide); the human growth
25   hormone (hGH) signal peptide genomic DNA sequence, which
is interrupted by the first intron of the hGH gene; and
the 3' untranslated sequence (UTS) of the hGH gene, which
contains a signal for polyadenylation.  Plasmid
pXAG-16 has the human cytomegalovirus immediate-early
30   (CMV IE) promoter and first intron (flanked by non-coding
exon sequences), while pXAG-28 is driven by the collagen
Iα2 promoter and also contains the β-actin gene's 5' UTS,
which contains the first intron of the β-actin gene.

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### A.   Cloning of the Complete α-Gal A
### cDNA, and Construction of the α-Gal A
### Expression Plasmid pXAG-16

The human α-gal cDNA was cloned from a human
5   fibroblast cDNA library that was constructed as follows.
Poly-A⁺ mRNA was isolated from total RNA, and cDNA
synthesis was performed using reagents for the
lambda ZapII® system according to the manufacturer's
instructions (Stratagene Inc., LaJolla, CA).  Briefly,
10  "first strand" cDNA was generated by reverse
transcription in the presence of an oligo-dT primer
containing an internal XhoI restriction endonuclease
site.  Following treatment with RNase H, the cDNA was
nick-translated with DNA polymerase I to generate double
15  stranded cDNA.  This cDNA was made blunt-ended with
T4 DNA polymerase, and ligated to EcoRI adaptors.  The
products of this ligation were treated with T4 DNA kinase
and digested with XhoI.  The cDNA was fractionated by
Sephacryl-400® chromatography.  Large and medium size
20  fractions were pooled and the cDNAs ligated to EcoRI and
XhoI-digested Lambda ZapII arms.  The products of this
ligation were then packaged and titered.  The primary
library had a titer of 1.2 x 10⁷ pfu/ml and an average
insert size of 925 bp.
25          A 210 bp probe from exon 7 of the human α-gal A
gene (Fig. 1, SEQ ID NO:19) was used to isolate the cDNA.
The probe itself was isolated from genomic DNA by the
polymerase chain reaction (PCR) using the following
oligonucleotides:  5'-CTGGGCTGTAGCTATGATAAAC-3' (Oligo 1;
30  SEQ ID NO:1) and 5'-TCTAGCTGAAGCAAAACAGTG-3' (Oligo 2;
SEQ ID NO:2).  The PCR product was then used to screen
the fibroblast cDNA library, and positive clones were
isolated and further characterized.  One positive clone,
phage 3A, was subjected to the lambda ZapII® system
35  excision protocol (Stratagene, Inc., La Jolla, CA),

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according to the manufacturer's instructions.  This
procedure yielded plasmid pBSAG3A, which contains the α-
gal A cDNA sequence in the pBluescriptSK-™ plasmid
backbone.  DNA sequencing revealed that this plasmid did

5 not contain the complete 5' end of the cDNA sequence.
Therefore, the 5' end was reconstructed using a PCR
fragment amplified from human genomic DNA.  To accomplish
this, a 268 bp genomic DNA fragment (Fig. 2, SEQ ID
NO:20) was amplified using the following

10 oligonucleotides:  5'-ATTGGTCCGCCCCTGAGGT-3' (Oligo 3;
SEQ ID NO:3) and 5'-TGATGCAGGAATCTGGCTCT-3' (Oligo 4;
SEQ ID NO:4).  This fragment was subcloned into a "TA"
cloning plasmid (Invitrogen Corp., San Diego, CA) to
generate plasmid pTAAGEI.  Plasmid pBSAG3A, which

15 contains the majority of the α-gal A cDNA sequence, and
pTAAGEI, which contains the 5' end of the α-gal A cDNA,
were each digested with SacII and NcoI.  The positions of
the relevant SacII and NcoI sites within the amplified
DNA fragment are shown in Fig. 2.  The 0.2 kb SacII-NcoI

20 fragment from pTAAGEI was isolated and ligated to
equivalently digested pBSAG3A.  This plasmid, pAGAL,
contains the complete α-gal A cDNA sequence, including
the sequence encoding the α-gal A signal peptide.  The
cDNA was completely sequenced (shown in Fig. 3 including

25 the α-gal A signal peptide; SEQ ID NO: 18) and found to
be identical to the published sequence for the human α-
gal A cDNA (Genbank sequence HUMGALA).

        The plasmid pXAG-16 was constructed via several
intermediates, as follows.  First, pAGAL was digested

30 with SacII and XhoI and blunt-ended.  Second, the ends of
the complete α-gal A cDNA were ligated to XbaI linkers
and subcloned into XbaI digested pEF-BOS (Mizushima et
al., Nucl. Acids Res. 18:5322, 1990), creating pXAG-1.
This construct contains the human granulocyte-colony

35 stimulating factor (G-CSF) 3' UTS and the human

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elongation factor-1α (EF-1α) promoter flanking the cDNA
encoding α-gal A plus the α-gal A signal peptide, such
that the 5' end of the α-gal A cDNA is fused to the EF-1α
promoter. To create a construct with the CMV IE promoter
5  and first intron, the α-gal A cDNA and G-CSF 3' UTS were
removed from pXAG-1 as a two kb XbaI-BamHI fragment. The
fragment was blunt-ended, ligated to BamHI linkers, and
inserted into BamHI digested pCMVflpNeo (which was
constructed as described below). The orientation was
10 such that the 5' end of the α-gal A cDNA was fused to the
CMV IE promoter region.

        pCMVflpNeo was created as follows. A CMV IE gene
promoter fragment was amplified by PCR using CMV genomic
DNA as a template and the oligonucleotides:
15 5'-TTTTGGATCCCTCGAGGACATTGATTATTGACTAG-3' (SEQ ID NO:23)
and 5'-TTTTGGATCCCGTGTCAAGGACGGTGAC-3' (SEQ ID NO:24).
The resulting product (a 1.6 kb fragment) was digested
with BamHI, yielding a CMV promoter-containing fragment
with cohesive BamHI-digested ends. The neo expression
20 unit was isolated from plasmid pMC1neopA (Stratagene
Inc., La Jolla, CA) as a 1.1 kb XhoI-BamHI fragment. The
CMV promoter-containing and neo fragments were inserted
into a BamHI-, XhoI-digested plasmid (pUC12). Notably,
pCMVflpNeo contains the CMV IE promoter region, beginning
25 at nucleotide 546 and ending at nucleotide 2105 (of
Genbank sequence HS5MIEP), and the neomycin resistance
gene driven by the Herpes Simplex Virus (HSV) thymidine
kinase promoter (the TKneo gene) immediately 5' to the
CMV IE promoter fragment. The direction of transcription
30 of the neo gene is the same as that of the CMV promoter
fragment. This intermediate construct was called pXAG-4.

        To add the hGH 3' UTS, the GCSF 3' UTS was removed
from pXAG-4 as an XbaI-SmaI fragment and the ends of
pXAG-4 were made blunt. The hGH 3' UTS was removed from
35 pXGH5 (Selden et al., Mol. Cellular Biol. 6:3173-3179,

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1986) as a 0.6 kb SmaI-EcoRI fragment.  After blunt-
ending this fragment, it was ligated into pXAG-4
immediately after the blunt-ended XbaI site of pXAG-4.
This intermediate was called pXAG-7.  The TKneo fragment
5   was removed from this plasmid as a HindIII-ClaI fragment
and the ends of the plasmid were blunted by "filling-in"
with the Klenow fragment of DNA polymerase I.  A neomycin
resistance gene driven by the SV40 early promoter was
ligated in as a blunted ClaI-BsmBI fragment from a digest
10  of pcDNeo (Chen et al., Mol. Cellular Biol. 7:2745-2752,
1987), placing the neo transcription unit in the same
orientation as the α-gal A transcription unit.  This
intermediate was called pXAG-13.

To complete pXAG-16, which has the 26 amino acid
15  hGH signal peptide coding sequence and first intron of
the hGH gene, a 2.0 kb EcoRI-BamHI fragment of pXAG-13
was first removed.  This fragment included the α-gal A
cDNA and the hGH 3' UTS.  This large fragment was
replaced with 3 fragments.  The first fragment consisted
20  of a 0.3 kb PCR product of pXGH5, which contains the hGH
signal peptide coding sequence and includes the hGH first
intron sequence, from a synthetic BamHI site located just
upstream of the Kozak consensus sequence to the end of
the hGH signal peptide coding sequence.  The following
25  oligonucleotides were used to amplify this fragment
(Fragment 1):  5'-TTTTGGATCCACCATGGCTA-3' (Oligo HGH101;
SEQ ID NO:5) and 5'-TTTTGCCGGCACTGCCCTCTTGAA-3'
(Oligo HGH102; SEQ ID NO:6).  The second fragment
consisted of a 0.27 kb PCR product containing sequences
30  corresponding to the start of the cDNA encoding the 398
amino acid α-gal A enzyme (i.e., lacking the α-gal A
signal peptide) to the NheI site.  The following
oligonucleotides were used to amplify this fragment
(Fragment 2): 5'-TTTTCAGCTGGACAATGGATTGGC-3' (Oligo AG10;
35  SEQ ID NO:7) and 5'-TTTTGCTAGCTGGCGAATCC-3' (Oligo AG11;

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SEQ ID NO:8). The third fragment consisted of the NheI-EcoRI fragment of pXAG-7 containing the remaining α-gal A sequence as well as the hGH 3' UTS (Fragment 3).

Fragment 1 (digested with BamHI and NaeI),

5 Fragment 2 (digested with PvuII and NheI), and Fragment 3 were mixed with the 6.5 kb BamHI-EcoRI fragment of pXAG-13 containing the neo gene and the CMV IE promoter and ligated together to generate plasmid pXAG-16 (Figure 4).

B. Construction of the α-Gal A

10     Expression Plasmid pXAG-28

The human collagen Iα2 promoter was isolated for use in the α-gal A expression construct pXAG-28 as follows. A 408 bp PCR fragment of human genomic DNA containing part of the human collagen Iα2 promoter was

15 isolated using the following oligonucleotides:

5'-TTTTGGATCCGTGTCCCATAGTGTTTCCAA-3'(Oligo 72;

SEQ ID NO:9) and 5'-TTTTGGATCCGCAGTCGTGGCCAGTACC-3'

(Oligo 73; SEQ ID NO:10).

This fragment was used to screen a human leukocyte

20 library in EMBL3 (Clontech Inc., Palo Alto, CA). One positive clone (phage 7H) containing a 3.8 kb EcoRI fragment was isolated and cloned into pBSIISK+ (Stratagene Inc., La Jolla, CA) at the EcoRI site (creating pBS/7H.2). An AvrII site was introduced in

25 pBSIISK+ by digesting with SpeI, which cleaves within the pBSIISK+ polylinker, "filling-in" with the Klenow fragment of DNA polymerase I, and inserting the oligonucleotide 5'-CTAGTCCTAGGA-3' (SEQ ID NO:11). This variant of pBSIISK+ was digested with BamHI and AvrII and

30 ligated to the 121 bp BamHI-AvrII fragment of the original 408 bp collagen Iα2 promoter PCR fragment described above, creating pBS/121COL.6.

The plasmid pBS/121COL.6 was digested with XbaI, which cleaves within the pBSIISK+ polylinker sequence,

35 "filled-in" with the Klenow fragment of DNA polymerase I,

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and digested with AvrII.  The 3.8 kb BamHI-AvrII fragment
of pBS/7H.2 was isolated and the BamHI site made
blunt-ended by treatment with Klenow enzyme.  The
fragment was then digested with AvrII and ligated to the
5    AvrII-digested vector, thus creating the collagen
promoter plasmid pBS/121bpCOL7H.18.

     Next the collagen promoter was fused to the 5' UTS
of the human β-actin gene, which contains the first
intron of the human β-actin gene.  To isolate this
10   sequence, a 2 kb PCR fragment was isolated from human
genomic DNA using the following oligonucleotides:
5'-TTTTGAGCACAGAGCCTCGCCT-3' (Oligo BA1; SEQ ID NO:12)
and
5'-TTTTGGATCCGGTGAGCTGCGAGAATAGCC-3' (Oligo BA2;
15   SEQ ID NO:13).

     This fragment was digested with BamHI and BsiHKAI
to release a 0.8 kb fragment containing the β-actin
5' UTS and intron.  A 3.6 kb SalI-SrfI fragment was then
isolated from the collagen promoter plasmid
20   pBS/121bpCOL7H.18 as follows.  pBS/121bpCOL7H.18 was
partially digested with BamHI (the BamHI site lies at the
5' end of the collagen Iα2 promoter fragment), made
blunt-ended by treatment with the Klenow fragment, and
ligated to a SalI linker (5'-GGTCGACC-3'), thereby
25   placing a SalI site upsteam of the collagen Iα2 promoter.
This plasmid was then digested with SalI and SrfI (the
SrfI site lies 110 bp upstream of the collagen Iα2
promoter CAP site), and the 3.6 kb fragment was isolated.
The 0.8 and 3.6 kb fragments were combined with SalI -
30   and BamHI - digested pBSIISK- (Stratagene Inc.,
La Jolla, CA), and a fragment composed of the following
four oligonucleotides annealed together (forming a
fragment with a blunt end and a BsiHKAI end):
5'-GGGCCCCCAGCCCCAGCCCTCCCATTGGTGGAGGCCCTTTTGGAGGCACCCTAG
35   GGCCAGGAAACTTTTGCCGTAT-3' (Oligo COL-1; SEQ ID NO:14),

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5'-AAATAGGGCAGATCCGGGCTTTATTATTTTAGCACCACGGCCGCCGAGAC
CGCGTCCGCCCCGCGAGCA-3' (Oligo COL-2; SEQ ID NO:15),
5'-TGCCCTATTTATACGGCAAAAGTTTCCTGGCCCTAGGGTGCCTCCAAAAGGGC
CTCCACCAATGGGAGGGCTGGGGCTGGGGGCCC-3' (Oligo COL-3;

5  SEQ ID NO:16), and 5'-CGCGGGGCGGACGCGGTCTCGGCGGCCGTGGTGCT
AAAATAATAAAGCCCGGATC-3' (Oligo COL-4; SEQ ID NO:17).
These four oligonucleotides, when annealed, correspond to
the region beginning at the SrfI site of the collagen
promoter and continuing through the BsiHKAI site of the

10 β-actin promoter.  The resulting plasmid was designated
pCOL/β-actin.

       To complete the construction of pXAG-28, the
SalI-BamHI fragment of pCOL/β-actin, containing the
collagen Iα2 promoter and β-actin 5' UTS, was isolated.

15 This fragment was ligated to two fragments from pXAG-16
(see Example 1A and Fig. 4):  (1) the 6.0 kb BamHI
fragment (containing the neo gene, plasmid backbone, the
cDNA encoding the 398 amino acid α-gal A enzyme, and the
hGH 3' UTS); and (2) the 0.3 kb BamHI-XhoI fragment

20 (which contains the SV40 poly A sequence from pcDneo).
pXAG-28 contains the human collagen Iα2 promoter fused to
the human β-actin 5' UTS, the hGH signal peptide (which
is interrupted by the hGH first intron), the cDNA
encoding the α-gal A enzyme, and the hGH 3' UTS.  A map

25 of the completed expression construct pXAG-28 is shown in
Fig. 5.

       C.  Transfection and Selection of Fibroblasts
       Electroporated with α-Gal A Expression Plasmids
       In order to express α-gal A in fibroblasts,

30 secondary fibroblasts were cultured and transfected
according to published procedures (Selden et al.,
WO 93/09222).

       The plasmids pXAG-13, pXAG-16 and pXAG-28 were
transfected by electroporation into human foreskin

35 fibroblasts to generate stably transfected clonal cell

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strains, and the resulting α-gal A expression levels were
monitored as described in Example ID. Secretion of α-
gal A by normal foreskin fibroblasts is in the range of
2-10 units/$10^6$ cells/24 hours. In contrast, the
5 transfected fibroblasts displayed mean expression levels
as shown in Table 1:


Table 1:        Mean α-gal A expression levels (+/- standard
                deviation)

---

10  pXAG-13:      420 +/- 344 U/$10^6$ cells/day
                  N=26 clonal strains
                  (range 3 - 1133 U/$10^6$ cells/day)


    pXAG-16:      2,051 +/- 1253 U/$10^6$ cells/day
                  N=24 clonal strains
15                (range 422 - 5200 U/$10^6$ cells/day)


    pXAG-28:      141 +/- 131 U/$10^6$ cells/day
                  N=38 clonal strains
                  (range 20 - 616 U/$10^6$ cells/day)


        These data show that all three expression
20  constructs are capable of increasing α-gal A expression
    many times that of nontransfected fibroblasts.
    Expression by fibroblasts stably transfected with pXAG-
    13, which encodes α-gal A linked to the α-gal A signal
    peptide, was substantially lower than expression by
25  fibroblasts transfected with pXAG-16, which differs only
    in that the signal peptide is the hGH signal peptide, the
    coding sequence of which is interrupted by the first
    intron of the hGH gene.

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Each time the transfected cells were passaged, the
secreted α-gal A activity was determined, the cells were
counted, and the cell density was calculated. Based on
the number of cells harvested and the time allowed for
5  secretion of α-gal A, the specific expression rate of α-
gal A was determined and is reported in Tables 2 and 3 as
secreted units (of α-gal A) per $10^6$ cells per 24 hour
period. Cell strains desirable for gene therapy or for
use in generation of material for purification of α-gal A
10 should display stable growth and expression over several
passages. Data from the cell strains shown in Tables 2
and 3, which were stably transfected with the α-gal A
expression construct pXAG-16, illustrate the fact that α-
gal A expression is stably maintained during serial
15 passage.

Table 2:    Growth and Expression of BRS-11 Cells
            Containing the α-Gal A Expression Construct
            pXAG-16.

| BRS-11 | | |
|---|---|---|
| Passage | Expression (units/$10^6$ cells/24 hr) | Cell Density (cells/$cm^2$) |
| 13 | 2601 | 4.80 x $10^4$ |
| 14 | 1616 | 4.40 x $10^4$ |
| 15 | 3595 | 4.40 x $10^4$ |

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Table 3:      Growth and Expression of HF503-242 Cells
              Containing the α-Gal A Expression Construct
              pXAG-16.

| HF503-242 | | |
|---|---|---|
| Passage | Expression (units/$10^6$ cells/24hr) | Cell Density (cells/$cm^2$) |
| 5 | 4069 | $2.80 \times 10^4$ |
| 6 | 7585 | $3.55 \times 10^4$ |
| 7 | 5034 | $2.48 \times 10^4$ |

### D.    Quantification of α-Gal A Expression

The activity of α-gal A activity was measured
using the water-soluble substrate 4-methylumbelliferyl-α-
D-galactopyranoside (4-MUF-gal; Research Products, Inc.)
by a modification of the protocol described by Ioannou et
al. (J. Cell Biol. 119:1137-1150, 1992). The substrate
was dissolved in substrate buffer (0.1 M citrate-
phosphate,
pH 4.6) to a concentration of 1.69 mg/ml (5 mM).
Typically, 10 μl of culture supernatant was added to 75
μl of the substrate solution. The tubes were covered and
allowed to incubate in a 37°C waterbath for 60 minutes.
At the end of the incubation period, 2 ml of glycine-
carbonate buffer (130 mM glycine, 83 mM sodium carbonate,
at pH 10.6), were used to stop the reaction. The
relative fluorescence of each sample was measured using a
model TKO100 fluorometer (Hoefer Scientific Instruments)
which has a fixed excitation wavelength of 365 nm and
detects a fixed emission wavelength of 460 nm. The

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readings of the samples were compared to standards
prepared from a 1 μM stock of methylumbelliferone (Sigma
Chemical Co.), and the amount of hydrolyzed substrate was
calculated.  The activity of α-gal A is expressed in
5  units; one unit of α-gal A activity is equivalent to one
nanomole of substrate hydrolyzed per hour at 37°C.  Cell
expression data were generally expressed as units of α-
gal A activity secreted/$10^6$ cells/24 hours.  This assay
was also used to measure the amount of α-gal activity in
10  cell lysates and in samples from various α-gal
purification steps, as discussed below.


Example II.  Purification of α-Gal A from the Conditioned
Medium of Stably Transfected Human Cell Strains
           Examples IIA-IIE illustrate that α-gal A may be
15  purified to near-homogeneity from the conditioned medium
of cultured human cell strains that have been stably
transfected to produce the enzyme.
           A.  Use of Butyl Sepharose[®] Chromatography
               as a First Step in the Purification of α-Gal A
20          Cold conditioned medium (1.34 liters) was
clarified by centrifugation and filtered through a
0.45 μm cellulose acetate filter using glass fiber
prefilters.  While stirring, the pH of the cold, filtered
medium was adjusted to 5.6 by the dropwise addition of
25  1 N HCl, and ammonium sulfate was added to a final
concentration of 0.66 M by the dropwise addition of a
stock solution (room temperature) of 3.9 M ultrapure
ammonium sulfate.  The medium was stirred for an
additional 5 minutes at 4°C, filtered as before, and
30  applied to a Butyl Sepharose[®] 4 Fast Flow column (81 ml
column volume, 2.5 x 16.5 cm; Pharmacia, Uppsala, Sweden)
that had been equilibrated in 10 mM MES-Tris, pH 5.6,
containing 0.66 M ammonium sulfate (buffer A).  The
chromatography was performed at 4°C on a Gradi-Frac[™]

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System (Pharmacia, Uppsala, Sweden) equipped with in-line
UV (280 nm) and conductivity monitors for assessing total
protein and salt concentration, respectively. After
sample application at a flow rate of 10 ml/min, the
5 column was washed with 10 column volumes of buffer A.
The α-gal A was eluted from the Butyl Sepharose® column
with a 14 column volume linear gradient from buffer A
(containing ammonium sulfate) to 10 mM MES-Tris, pH 5.6
(no ammonium sulfate). Fractions were assayed for
10 α-gal A activity by the 4-MUF-gal assay, and those
containing appreciable enzyme activity were pooled. As
seen in Fig. 6 and the purification summary (Table 3),
this step removes approximately 99% of the contaminating
protein (pre-column sample=8.14 g total protein; post-
15 column sample=0.0638 g total protein).

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**Table 4:    Purification of α-Gal A from the Conditioned
Medium of Stably Transfected Human Fibroblasts**

| Purificat ion Step | Volum e (ml) | α-Gal A Activi ty (x $10^6$ Units) | Tota l Prot ein (mg) | Specific Activity (x $10^6$ Units /mg) | Fold Purifica tion (Cumulat ive) | Percen t Recove ry |
|---|---|---|---|---|---|---|
| Culture supernata nt | 1340 | 14.6 | 8140 | 0.0018 | ≈1 | ≈100 |
| Butyl Sepharose ® | 417 | 14.1 | 63.8 | 0.221 | 123 | 96.6 |
| Heparin Sepharose ® | 134 | 12.1 | 14.6 | 0.829 | 436 | 82.9 |
| Hydroxyap atite | 47 | 9.73 | 4.46 | 2.18 | 1220 | 66.6 |
| Q Sepharose ® | 31.5 | 8.91 | 3.31 | 2.69 | 1503 | 61.0 |
| Superdex® 200 | 10 | 8.58 | 2.93 | 2.92 | 1634 | 59.0 |

**B.    Use of Heparin Sepharose® Chromatography
as a Step for Purification of α-Gal A**

The Butyl Sepharose® column peak fractions were
dialyzed at 4°C against (4 liters) of 10 mM MES-Tris,
pH 5.6 (changed once). The conductivity of the dialysate
was adjusted to 1.0 mMHO at 4°C by addition of $H_2O$ or
NaCl as necessary. Afterward, the sample was applied to
a column of Heparin Sepharose® 6 Fast Flow (Pharmacia,
Uppsala, Sweden; 29 ml column volume, 2.5 x 6 cm) that
had been equilibrated in 10 mM MES-Tris, pH 5.6,
containing 9 mM NaCl (buffer B). This was done at 4°C at
a flow rate of 10 ml/min. In-line UV (280 nm) and

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conductivity monitors measured total protein and salt
concentration. After the sample was applied, the column
was washed with 10 column volumes of buffer B followed by
a 3 column volume linear gradient to 8% buffer C/92%
5  buffer B (where buffer C is 10 mM MES-Tris, pH 5.6,
containing 250 mM NaCl) and a 10 column volume wash with
8% buffer C. This was followed by elution of α-gal A
with a 1.5 column volume linear gradient to 29% buffer C
and a subsequent 10 column volume linear gradient to
10  35% buffer C. Fractions were assayed for α-gal A
activity, and those containing appreciable activity were
pooled.

     C.   Use of Hydroxyapatite
          Chromatography as a Step for
15        Purification of α-Gal A
     The heparin pool was filtered and applied
directly to a column of Ceramic Hydroxyapatite HC (40 μm;
American International Chemical, Natick, MA; 12 ml column
volume, 1.5 x 6.8 cm) that had been equilibrated in 1 mM
20  sodium phosphate, pH 6.0 (buffer D). The chromatography
was performed at room temperature on a hybrid Gradi-
Frac"/FPLC® System (Pharmacia, Uppsala, Sweden) equipped
with in-line UV (280 nm) and conductivity monitors.
After the sample was applied (5 ml/min), the column was
25  washed with 10 column volumes of buffer D. The α-gal A
was eluted with a 7 column volume linear gradient to 42%
buffer E/58% buffer D (where buffer E is 250 mM sodium
phosphate, pH 6.0) followed by a 10 column volume
gradient to 52% buffer E. Fractions were assayed for α-
30  gal A activity, and the fractions containing appreciable
activity were pooled.

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### D.   Use of Q Sepharose® Anion Exchange Chromatography as a Step for Purification of α-Gal A

The hydroxyapatite pool was diluted

5  approximately 1.5 fold with $H_2O$ to a final conductivity
   of 3.4-3.6 mMHO at room temperature.  After filtering,
   the sample was applied to a column of Q Sepharose® HP
   (Pharmacia, Uppsala, Sweden; 5.1 ml column volume,
   1.5 x 2.9 cm) equilibrated in 10% buffer G/90% buffer F,

10  where buffer F is 25 M sodium phosphate, pH 6.0, and
   buffer G is 25 mM sodium phosphate, pH 6.0, 250 mM NaCl.
   The chromotography was performed at room temperature on
   the Gradi-Frac™/FPLC® hybrid system (Pharmacia, Uppsala,
   Sweden), and total protein and salt concentrations were

15  monitored by the in-line monitors.  The sample was
   applied at a flow rate of 5 ml/min, then the following
   steps were performed:   (1) a 5 column volume wash at
   10% buffer G, (2) a 7 column volume wash at 12% buffer G,
   (3) a 3 column volume linear gradient to 50% buffer G,

20  (4) a 10 column volume linear gradient to 53% buffer G,
   (5) a 3 column volume gradient to 100% buffer G, and
   (6) a 10 column volume wash at 100% buffer G.  The α-
   gal A eluted primarily during steps 3 and 4.  Fractions
   containing appreciable activity were pooled (the

25  "Q pool").

### E.   Use of Superdex®-200 Gel Filtration Chromatography as a Step for Purification of α-Gal A

The Q pool was concentrated approximately 5-

30  fold using Centriprep®-10 centrifugal concentrator units
   (Amicon, Beverly, MA), and applied to a column of
   Superdex® 200 (Pharmacia, Uppsala, Sweden; 189 ml column
   volume, 1.6 x 94 cm).  The column was equilibrated and
   eluted with 25 mM sodium phosphate, pH 6.0, containing

35  150 mM NaCl.  The chromatography was performed on an

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FPLC® system (Pharmacia, Uppsala, Sweden) at room
temperature using an in-line UV monitor (280 nm) to
follow elution of the protein. The volume of the sample
applied to the column was ≤ 2 ml, the flow rate was 0.5
5  ml/min, and the fraction size was 2 ml. Multiple column
runs were performed; fractions were assayed for α-gal A
activity and fractions containing appreciable activity
were pooled.

          The pooled fractions from the Superdex® 200
10 column were concentrated using Centriprep-10 units,
aliquoted, snap-frozen, and stored at -80°C for short
periods of time. A summary of this example of α-gal A
purification is shown in Table 3. The final yield of α-
gal A was 59% of the starting material activity, and the
15 specific activity of the purified product was
2.92 x $10^6$ units/mg protein. The resulting product
showed a high level of purity after electrophoresis under
reducing conditions on a 4-15% SDS-polyacrylamide gel,
which was subsequently silver-stained.


20 <u>Example III.   Formulation and Storage of Purified α-Gal A</u>
          Highly purified α-gal A is not stable for
extended periods of time when stored as a dilute solution
of purified protein (≤1 mg protein/ml). Therefore, a
formulation was devised to improve stability during
25 prolonged storage, i.e. storage lasting several weeks to
at least several months. The purified enzyme was
concentrated to at least 1 mg/ml using a centrifugal
concentrator (in enzyme buffer consisting of 25 mM sodium
phosphate (pH 6.0) and 150 mM NaCl). Human serum albumin
30 (HSA; Buminate®, Baxter-Hyland) was added to a final
concentration of 2.5 mg/ml. The protein solution was
then sterile filtered using a 0.2 μm cellulose acetate
filter (Schleicher and Schuell) attached to a syringe.
The α-gal A solution was dispensed into sterile, pyrogen-

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free glass vials, sealed with a Teflon cap, snap-frozen, and stored at -20°C.

Stability of the α-gal A activity was evaluated over a three month period using the 4-MUF-gal assay. The data presented in Table 5 demonstrate that there was no loss of enzyme activity over the test period. The acidic pH of the formulation (< 6.5) is critical to the stability of the highly purified enzyme.

Table 5:   Stability of Formulated α-Gal A at -20°C

| Sample | Specific Activity (Units/mg total protein) |
|--------|--------------------------------------------|
| time 0 | $2.24 \times 10^6$ +/- $0.33 \times 10^6$ |
| week 1 | $2.40 \times 10^6$ +/- $0.25 \times 10^6$ |
| week 2 | $2.42 \times 10^6$ +/- $0.21 \times 10^6$ |
| week 3 | $2.37 \times 10^6$ +/- $0.05 \times 10^6$ |
| month 1 | $2.39 \times 10^6$ +/- $0.16 \times 10^6$ |
| month 2 | $2.31 \times 10^6$ +/- $0.26 \times 10^6$ |
| month 3 | $2.29 \times 10^6$ +/- $0.17 \times 10^6$ |

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Example IV.   α-Gal A Produced by Human Cell Strains is
Suitable for Treatment of α-Gal A Deficiency

      The structural and functional properties of
purified human α-gal A prepared in accordance with the
5  invention were investigated in order to demonstrate that
the DNA molecules described herein and the corresponding
expressed glycoproteins produced by transfected human
cell strains can be used in gene or enzyme replacement
therapies, respectively.

10       A.   Size of α-Gal A Produced by Stably
Transfected   Human Cells in Culture

      The molecular mass of α-gal A was estimated by
MALDI-TOF mass spectrometry.  These results demonstrate
that the molecular mass of the dimer is 102,353 Da, while
15  that of the monomer is 51,002 Da.  The expected molecular
mass of the monomer, based on amino acid composition, is
45,400 Da.  Therefore, it can be inferred that the
carbohydrate content of the enzyme accounts for up to
5,600 Da of the molecular  weight.

20      The results of standard amino acid analysis
performed on the purified protein are consistent with the
conclusion that the protein produced by transfected human
cells is identical to the protein purified from human
tissues at the amino acid level.

25       B.  N-Terminal Processing of α-Gal A
Produced by Stably Transfected Human Cells

      The human α-gal A cDNA nucleotide  sequence
encodes 429 amino acids.  The 31 N-terminal amino acids
constitute a signal peptide sequence, which is cleaved as
30  the nascent protein transits the endoplasmic reticulum
membrane (LeDonne et al., Arch. Biochem. Biophys.
224:186, 1983; Lemansky et al., J. Biol. Chem. 262:2062,
1987).  In order to confirm that α-gal A is properly
processed when associated with an heterologous signal
35  peptide sequence (for example, the human growth hormone

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signal sequence) and expressed in transfected human
fibroblasts, ten N-terminal amino acids of the secreted
protein were microsequenced.  Samples were
electrophoresed by SDS-PAGE and transferred to
5    ProBlott® (ABI, Foster City, CA) using a 10 mM CAPS
(pH 11.0), 10% methanol buffer system.  The protein on
the ProBlott® was visualized by Coomassie staining, and
an appropriately sized (50 kDa) band was excised.  N-
terminal sequence was obtained using an Applied
10   Biosystems pulse-liquid phase amino acid sequenator that
performs automated Edman degradation.  The N-terminal
sequence obtained, LDNGLARTPT (SEQ ID NO:28), is
consistent with proper cleavage of the signal peptide and
matches the N-terminal sequence predicted for the
15   secreted protein.

              C.   C-Terminal Amino Acid of α-Gal A
         Produced by Stably Transfected Human Cells
              The C-terminal amino acid residue of secreted
α-gal A produced in accordance with the invention was
20   identified using an automated Hewlett Packard C-terminal
sequencer.  The results indicated a leucine residue at
the C-terminus, which agrees with the C-terminal amino
acid predicted by the DNA sequence.

              D.   Carbohydrate Modification of α-Gal A
25               Produced by Stably Transfected Human Cells
              The glycosylation pattern of α-gal A produced
in accordance with the invention was also evaluated.
Proper glycosylation is important for optimal in vivo
activity of α-gal A; α-gal A expressed in non-
30   glycosylating systems is inactive or unstable
(Hantzopolous et al., Gene 57:159, 1987).  Glycosylation
is also important for the internalization of α-gal A into
the desired target cells, and affects the circulating
half-life of the enzyme in vivo.  On each subunit of α-
35   gal A there are four sites available for addition of

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asparagine-linked carbohydrate chains, of which only
three are occupied (Desnick et al., In The Metabolic and
Molecular Bases of Inherited Disease, pp 2741-2780,
McGraw Hill, New York, 1995).

5        A sample of α-gal A produced by stably
transfected cells was treated with neuraminidase, which
is isolated from A. urafaciens, (Boehringer-Mannheim,
Indianapolis, IN) to remove sialic acid. This reaction
was performed by treating 5 μg of α-gal A overnight with
10   10 mU of neuraminidase at room temperature in a total
volume of 10 μl of acetate buffered saline (ABS, 20 mM
sodium acetate, pH. 5.2, 150 mM NaCl).

Purified α-gal A produced by stably transfected
cells was also dephosphorylated using alkaline
15   phosphatase (calf intestinal alkaline phosphatase,
Boehringer-Mannheim, Indianapolis, IN), by treating 5 μg
of α-gal A overnight at room temperature with 15 U of
alkaline phosphatase in ABS (pH raised to 7.5 with 1 M
Tris).

20       The samples were analyzed by Western blot with
an α-gal A-specific antibody. The antibody used was a
rabbit polyclonal anti-peptide antibody, which was
produced using a peptide representing amino acids 68-81
of α-gal A as an immunogen. Following transfer of the
25   protein to PVDF (Millipore, Bedford, MA), the membrane
was probed with a 1:2000 dilution of the anti-serum in
2.5% blotto (non-fat dry milk in 20 mM Tris-HCl, pH 7.5,
0.05% Tween-20). This was followed by detection with
goat anti-rabbit IgG conjugated to horseradish peroxidase
30   (Organon Teknika/Cappel, Durham, NC; 1:5000 dilution) and
reagents of the ECL chemiluminescence kit (Amersham,
Arlington Heights, IN).

Treatment of α-gal A with neuraminidase results
in a slight shift in molecular mass (approximately 1500-
35   2000 Da or 4-6 sialic acids/monomer), suggesting that

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there is extensive modification of α-gal A with sialic
acid. For reference, the plasma form of α-gal A has 5-6
sialic acid residues per monomer, and the placental form
has 0.5-1.0 sialic acid residues per monomer (Bishop
5  et al., J. Biol. Chem. 256:1307, 1981).

Another method used to examine the sialic acid
and mannose-6-phosphate modifications of α-gal A was
isoelectric focusing (IEF), where the samples are
separated on the basis of their isoelectric point (pI) or
10 net charge. Thus, removal of charged residues such as
sialic acid or phosphate from α-gal A would be expected
to alter the mobility of the protein in the IEF system.

To perform the IEF experiment, samples of α-
gal A produced in accordance with the invention were
15 treated with neuraminidase and alkaline phosphatase,
mixed 1:1 with 2X Novex sample buffer (with 8 M urea,
pH 3.0-7.0), and loaded onto a 6 M urea IEF gel
(5.5% polyacrylamide) made using Pharmalyte® (Pharmacia,
Uppsala, Sweden; pH 3.0-6.5; Pharmalyte® 4-6.5 and 2.5-
20 5.5, 0.25 ml each per gel). Isoelectric point standards
(Bio-Rad) were also included. Following electrophoresis,
the gel was transferred to PVDF, and Western blot
analysis performed as described above.

The α-gal A produced by stably transfected
25 human fibroblasts consisted of three major isoforms with
a pI range of approximately 4.4-4.65. These values are
similar to the pIs of the plasma and splenic forms of
α-gal A (Bishop et al., J. Biol. Chem. 256:1307, 1981).
Neuraminidase treatment of the enzyme increased the pI of
30 all three isoforms, indicating that all were modified to
some extent by sialic acid. These data suggest that the
α-gal A produced by stably transfected human cells should
have a desirable plasma half-life, indicating that this
material is well suited for pharmacologic use. Further,
35 treatment of neuraminidase-treated α-gal A with alkaline

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phosphatase further increased the pI of a portion of the
protein to approximately 5.0-5.1, indicating that the
enzyme bears one or more mannose-6-phosphate residues.
This modification is significant in that it is required
for efficient internalization of α-gal A by the target
cells.

### E.  Specific Activity of α-gal A
### Purified from Stably Transfected Fibroblasts

The potency or specific activity of purified
α-gal A is calculated by measuring both the catalytic
activity of the enzyme (with the 4-MUF-gal assay), and
the protein concentration.  The protein concentration can
be determined by any standard method, such as with the
BCA system (Pierce), or by measuring the absorbance at
280 nm and using the mg/ml extinction coefficient of 2.3
(determined from amino acid analysis) to calculate the
value.  Using these techniques, the specific activity of
α-gal A purified from the conditioned medium of
transfected human fibroblasts is 2.2-2.9 x $10^6$ units/mg
of protein, which is comparable to the specific activity
of α-gal A that is purified from human tissues (Bishop
et al., J. Biol. Chem. 256:1301, 1981).

### F.  Mannose or Mannose-6-Phosphate Mediated
### Internalization of α-Gal A

In order for the α-gal A produced by stably
transfected cells to be an effective therapeutic agent
for α-gal A deficiencies, the enzyme must be internalized
by the affected cells.  α-Gal A is not active at
physiological pH levels, and is unlikely to be effective
in the blood or interstitial fluids.  It metabolizes
accumulated lipid substrates optimally only when
internalized in the acidic environment of the lysosome.
This internalization is mediated by the binding of α-
gal A to mannose-6-phosphate (M6P) receptors, which are
expressed on the cell surface and deliver the enzyme to

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the lysosome via the endocytic pathway. The M6P receptor
is ubiquitously expressed; most somatic cells express it
to some extent. The mannose receptor, which is specific
for exposed mannose residues on glycoproteins, is less
5 prevalent. The latter receptors are generally found only
on macrophage and macrophage-like cells, and provide an
additional means of α-gal A entry into these cell types.

In order to demonstrate M6P-mediated
internalization of α-gal A, skin fibroblasts from a Fabry
10 disease patient (NIGMS Human Genetic Mutant Cell
Repository) were cultured overnight in the presence of
increasing concentrations of purified α-gal A of the
invention. Some of the samples contained 5 mM soluble
M6P, which competitively inhibits binding to, and as a
15 result, internalization by, the mannose-6 phosphate
receptor. Other samples contained 30 μg/ml mannan, which
inhibits binding to, and as a result, internalization by,
the mannose receptor. Following incubation, the cells
were washed and harvested by scraping into lysis buffer
20 (10 mM Tris, pH 7.2, 100 mM NaCl, 5 mM EDTA, 2 mM
Pefabloc" (Boehringer-Mannheim, Indianapolis, IN) and
1% NP-40). The lysed samples were then assayed for
protein concentration and α-gal A activity. The results
are expressed as units of α-gal A activity/mg cell
25 protein. The Fabry cells internalized α-gal A in a dose-
dependent manner (Fig. 7). This internalization was
inhibited by mannose-6 phosphate, but there was no
inhibition with mannan. Therefore, internalization of
α-gal A in Fabry fibroblasts is mediated by the mannose-6
30 phosphate receptor, but not by the mannose receptor.

α-gal A is also internalized *in vitro* by
endothelial cells, important target cells for the
treatment of Fabry disease. Human umbilical vein
endothelial cells (HUVECs) were cultured overnight with
35 7500 units of α-gal A; some of the wells contained M6P.

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After the incubation period, cells were harvested and assayed for α-gal A as described above. The cells incubated with α-gal A only had enzyme levels almost 10-fold above those of control (no incubation with α-gal A)

5  cells. M6P inhibited the intracellular accumulation of α-gal A, suggesting that the internalization of α-gal A by HUVECs is mediated by the M6P receptor. Thus, the human α-gal A of the invention is internalized by clinically relevant cells.

10        Few cultured human cell lines are known to express the mannose receptor. However, a mouse macrophage-like cell line (J774.E) which bears mannose receptors but few if any mannose 6-phosphate receptors can be used to determine whether purified α-gal A of the

15  invention is internalized via the mannose receptor (Diment et al., J. Leukocyte Biol. 42:485-490, 1987). J774.E cells were cultured overnight in the presence of 10,000 units/ml α-gal A. Selected samples also contained 2 mM M6P, and others contained 100 μg/ml mannan. The

20  cells were washed and harvested as described above, and the total protein and α-gal A activity of each sample was determined. The results are shown in Table 5. M6P does not inhibit the uptake of α-gal A by these cells, while mannan decreases the accumulated α-gal A levels by 75%.

25  Thus, the α-gal A of the invention may be internalized by the mannose receptor in cell types that express this particular cell surface receptor.

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Table 6.  Internalization of α-Gal A by J774.E Cells.

**α-Gal A Activity (units/mg total protein)**

|         | No additions | + α-gal A | + α-gal A, + M6P | +α-gal A, + mannan |
|---------|--------------|-----------|------------------|--------------------|
| J774.E  | 409±25       | 6444±554  | 6297±674         | 1654±323           |

[mannan] = 100 μg/ml
5  [M6P] = 2 mM or 660 μg/ml


These experiments demonstrate that the α-gal A
produced by stably transfected human cells may be
internalized by cells via the mannose or mannose-6-
phosphate receptor.

10       G.  Correction of Fabry Fibroblasts by Human
Fibroblasts Expressing α-Gal A
         For gene therapy, an implant of autologous
cells producing α-gal A must produce the enzyme in a form
modified appropriately to "correct" the α-gal A
15 deficiency in target cells.  To assess the effect of α-
gal A production by transfected human fibroblasts on
Fabry cells, fibroblasts harvested from Fabry disease
patients (NIGMS Human Genetics Mutant Cell Repository)
were co-cultured with an α-gal A-producing cell strain
20 (BRS-11) in Transwells® (Costar, Cambridge, MA).  The
experimental scheme is depicted in Fig. 8.  Fabry cells
were cultured in 12-well tissue culture dishes, some of
which contained inserts (Transwells®, 0.4 μm pore size)
having a surface on which cells can be grown.  The growth
25 matrix of the insert is porous and allows macromolecules
to pass from the upper to the lower milieu.  One set of
inserts contained normal human foreskin (HF) fibroblasts,

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which secrete minimal levels of α-gal A, while another
set contained the stably transfected human fibroblast
strain, BRS-11, which secretes large amounts of α-gal A.
In the wells co-cultured with α-gal A-producing cells, α-
5  gal A can enter the medium bathing the Fabry cells, and
potentially be internalized by the Fabry cells.

The data in Table 7 show that Fabry cells
internalized the secreted α-gal A.  The intracellular
levels of α-gal A were monitored for three days.  Those
10  cells cultured alone (no insert) or in the presence of
non-transfected foreskin fibroblasts (HF insert) had very
low intracellular levels of α-gal A activity.  The Fabry
cells cultured with the α-gal A-producing (BRS-11 insert)
cells, however, exhibited enzyme levels similar to those
15  of normal cells by the end of Day 2 (normal fibroblasts
have 25-80 units α-gal A/mg protein).  That the
correction is attributable to α-gal A taken up via the
M6P receptor is demonstrated by its inhibition with
mannose-6-phosphate (BRS-11 insert + M6P).

20  Table 7.  Correction of Fabry Fibroblasts by Human
              Fibroblasts Expressing α-Gal A


          α-Gal A Activity (units/mg total protein)

| Time | no insert | HF insert | BRS-11 insert | BRS-11 insert+M6P |
|------|-----------|-----------|---------------|-------------------|
| Day 1 | 2 ± 1 | 2 ± 1 | 13 ± 1 | 4 ± 1 |
| Day 2 | 2 ± 1 | 2 ± 1 | 40 ± 11 | 6 ± 2 |
| Day 3 | 2 ± 1 | 5 ± 1 | 85 ± 1 | 9 ± 1 |

H. Utility of Other Cell Types

Other cell types can be used in the method
described herein.  The cells can be obtained from a
30  variety of tissues and include all cell types that can be

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maintained in culture.  For example, primary and
secondary cells that can be transfected by the present
method include human fibroblasts, keratinocytes,
epithelial cells (e.g., mammary or intestinal epithelial
5  cells), endothelial cells, glial cells, neural cells,
formed elements of the blood (e.g., lymphocytes and bone
marrow cells), muscle cells, and precursors of these
somatic cell types.  Fibroblasts are of particular
interest.  Primary cells are preferably obtained from the
10 individual to whom the transfected primary or secondary
cells are to be administered, so that they will not be
rejected by the patient's immune system.  However, if
proper attention is paid to avoiding or suppressing
immunorejection (as described below), cells of a human
15 donor other than the patient can be used as well.  This
would permit use of cells from an standardized,
established, stably transfected cell line in all
patients.

       <u>I.  Administration of α-Gal A-Expressing Cells</u>
20          The cells described above may be introduced
into an individual, through various standardized routes
of administration, so that they will reside in, for
example, the renal subcapsule, a subcutaneous
compartment, the central nervous system, the intrathecal
25 space, the liver, the intraperiotoneal cavity, or within
a muscle.  The cells may also be injected intravenously
or intra-arterially so that they circulate within the
individual's bloodstream.  Once implanted in the
individual, the transfected cells will produce and.
30 secrete the therapeutic product, glycosylated human α-gal
A.

          The number of genetically modified cells that
will be introduced into the individual will vary, but can
be determined by skilled artisans.  The age, weight, sex,
35 and general physical condition of each patient, as well

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as the volume of distribution, the half-life and
bioavailability of the enzyme, and the *in vivo*
productivity of the genetically modified cells, will be
among the primary considerations in determining dosage
5  and route of administration.  Typically, between one
million and one billion cells will be used, with
expression levels ranging from 100-100,000 units per $10^6$
cells per day.  If necessary, the procedure may be
repeated or modified until the desired result, for
10  example, relief from the symptoms associated with Fabry
disease, is achieved.

    As described above, the cells used will
generally be patient-specific, i.e., obtained from the
individual to whom the transfected primary or secondary
15  cells are to be administered, so that they will not be
rejected by the patient's immune system.  If, however,
this scenario is not possible or desirable, cells may be
obtained from another individual, genetically modified as
described herein, and implanted into the patient who is
20  suffering from α-gal A deficiency.

    The use of cells from an individual other than
the recipient might require administration of an
immunosuppressant, alteration of histocompatibility
antigens, or use of a barrier device to prevent rejection
25  of the implanted cells.  The barrier device will be made
of a material (e.g., a membrane such as XM-50 from
Amicon, Beverly, MA) that permits the secreted product to
pass into the recipient's circulation or tissues, but
prevents contact between the implanted cells and the
30  recipient's immune system, and thus prevents an immune
response to (and possible rejection of) the cells by the
recipient.  For further guidance regarding gene therapy,
see Selden et al. (WO 93/09222).

    The cells may alternatively be embedded in a
35  matrix or gel material, such as described in co-owned

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U.S.S.N. 08/548/002, which describes the use of hybrid
matrix implants, or in Jain et al. (PCT application
WO 95/19430), which describes macroencapsulation of
secretory cells in a hydrophilic gel material (each of
5  which is hereby incorporated by reference).

      J. Pharmaceutical Formulation for Conventional
      Administration of α-Gal A Protein

      The α-gal A protein that is expressed and
secreted by stably transfected (or otherwise genetically
10  modified) human cells and purified as described herein
may be administered to individuals who produce
insufficient or defective α-gal A protein.  The protein
may be administered in a pharmaceutically acceptable
carrier, at a pH below 6.5, e.g. in a formulation as
15  described in Example III.  Examples of excipients which
may be included with the formulation are buffers such as
citrate buffer, phosphate buffer, acetate buffer, and
bicarbonate buffer, amino acids, urea, alcohols, ascorbic
acid, phospholipids, proteins, such as serum albumin and
20  gelatin, EDTA, sodium chloride, liposomes,
polyvinylpyrollidone, mannitol, sorbitol, glycerol,
propylene glycol and polyethylene glycol (e.g., PEG-4000,
PEG-6000).  The route of administration may be, for
example, intravenous, intra-arterial, subcutaneous,
25  intraperitoneal, intracerebral, intramuscular,
intrapulmonary, or transmucosal.  The route of
administration and the amount of protein delivered will
be determined by factors that are well within the ability
of skilled artisans to assess.  Furthermore, skilled
30  artisans are aware that the route of administration and
dosage of a therapeutic protein may be varied for a given
patient until a therapeutic dosage level is obtained.
Typically, doses of α-gal A of 0.01-100 mg/kg of body
weight will be administered.  It is expected that

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regularly repeated doses of the protein will be necessary
over the life of the patient.

     K. Treatment of Other Conditions
     Caused by Enzyme Deficiencies

5        It is likely that other conditions caused by
deficiencies in lysosomal storage enzymes other than
α-gal A will be amenable to treatment by methods
comparable to those described herein. In these cases,
DNA which encodes a functional form of the deficient
10 enzyme would be substituted for the DNA encoding α-gal A
in the expression constructs disclosed herein. Examples
of enzyme deficiency syndromes that have been identified,
and that may be amenable to treatment as described
herein, are shown in Table 8. The information in this
15 Table is taken from E. Neufeld (Ann. Rev. Biochem.
60:257-280, 1991), which is hereby incorporated by
reference.


Table 8. Summary of Lysosomal Storage Disorders

---

| Disorder | Primary deficiency [secondary deficiency] |
|---|---|
| **Disorders of sphingolipid degradation** | |
| Fabry disease | α-galactosidase |
| Farber disease | ceramidase |
| Gaucher disease | glucocerebrosidase |
| G$_{M1}$ gangliosidosis | β-galactosidase |

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| $G_{M2}$ gangliosidoses subunit | $\beta$-hexosaminidase, $\alpha$- |
|---|---|
|    Tay-Sachs disease | [hexosaminidase A] |
|    Sandhoff disease 5 subunit | $\beta$-hexosaminidase, $\beta$- |
| | [hexosaminidases A and B] |
|    Activator deficiency Krabbe disease | $G_{M2}$ activator galactosylceramidase |
| Metachromatic leukodystrophy 10   enzyme-deficient form   activator-deficient form activator/saposin | arylsulfatase A sulfatide |
|    Mucolipidosis IV | primary defect unknown [ganglioside sialidase] |
| 15 Multiple sulfatase deficiency sulfatases] | primary defect unknown [deficiency of all |
|    Niemann-Pick disease | sphingomyelinase |
|    Schindler disease 20 acetylgalactosaminidase | $\alpha$-N- |

<u>Disorders of glycoprotein degradation</u>

| Aspartylglycosaminuria | aspartylglycosaminidase |
|---|---|
| Fucosidosis | $\alpha$-L-fucosidase |

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| Galactosialidosis protein/cathepsin | protective |
| --- | --- |

[β-galactosidase and

sialidase]

| 5  α-Mannosidosis | α-mannosidase |
| --- | --- |
| β-Mannosidosis | β-mannosidase |
| *Sialidosis* | sialidase |

## Disorders of glycosaminoglycan degradation

| Hunter syndrome | iduronate sulfatase |
| --- | --- |
| 10  Hurler and Scheie syndromes | α-L-iduronidase |
| Maroteaux-Lamy syndrome | GalNAc 4- sulfatase/arylsulfatase |
| Morquio syndrome | |
| A-subtype | Gal 6-sulfatase |
| 15  B-subtype | β-galactosidase |
| Sanfilippo syndrome A-subtype | heparan *N*-sulfatase |
| B-subtype | α-*N*-acetylglucosaminidase |
| C-subtype | AcetylCoA:  glucosamine |
| 20 | *N*-acetyltransferase |

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D-subtype                          GlcNAc 6-sulfatase

Sly syndrome                       β-glucuronidase

<u>Other single enzyme deficiency disorders</u>

Pompe disease (glycogenosis II)   α-glucosidase

5 Wolman disease                   acid lipase

Disorders of lysosomal enzyme biosynthesis

I-cell disease and pseudoHurler   6-phospho-*N*-

acetylglucosamine

                                   transferase

10   polydystrophy                 [mislocalization of many

lysosomal

                                   enzymes]

<u>Disorders of lysosmal membrane transport</u>

Cystinosis                         cystine transport

15 Sialic storage and Salla diseasesialic acid transport

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### V.  Other Embodiments

The invention described herein has been
exemplified in part by methods of treatment that employ
cells which express a particular gene product following
5   transfection, i.e., after introduction of a construct
encoding the gene product and having regulatory elements
that control expression of the coding sequence.  These
methods may also be carried out using cells that have
been genetically modified by other procedures, including
10  gene targeting and gene activation (see Treco et al.
(WO 95/31560, herein incorporated by reference; see also
Selden et al. WO 93/09222).

The hGH signal peptide can be used with
heterologous proteins other than $\alpha$-gal A, to increase the
15  level of expression and secretion of the heterologous
protein.  Examples of such proteins include $\alpha$-1
antitrypsin, antithrombin III, apolipoprotein E,
apolipoprotein A-1, blood clotting factors V, VII, VIII,
IX, X, and XIII, bone growth factor-2, bone growth
20  factor-7, calcitonin, catalytic antibodies, DNAse,
erythropoietin, FSH-$\beta$, globins, glucagon,
glucocerebrosidase, G-CSF, GM-CSF, growth hormone, immune
response modifiers, immunoglobulins, insulin,
insulinotropin, insulin-like growth factors,
25  interferon-$\beta$, interferon-$\beta$ nerve growth factors,
interleukin-1, interleukin-2, interleukin-3, interleukin-
4, interleukin-6, interleukin-11, interleukin-12, IL-2
receptor, IL-1 receptor antagonists, low density
lipoprotein receptor, M-CSF, parathyroid hormone, protein
30  kinase C, soluble CD4, superoxide dismutase, tissue
plasminogen activator, TGF-$\beta$, tumor necrosis factor,
TSH$\beta$, tyrosine hydroxylase, and urokinase.

Other embodiments are within the following claims.

50

## SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Transkaryotic Therapies, Inc.

    (ii) TITLE OF INVENTION: THERAPY FOR α-GALACTOSIDASE A
        DEFICIENCY

    (iii) NUMBER OF SEQUENCES: 28

    (iv) CORRESPONDENCE ADDRESS:
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    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 08/712,614
        (B) FILING DATE: 13-SEP-1996
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Fraser, Janis K.
        (B) REGISTRATION NUMBER: 34,819
        (C) REFERENCE/DOCKET NUMBER: 07236/003WO1

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 617/542-5070
        (B) TELEFAX: 617/542-8906
        (C) TELEX: 200154

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGGGCTGTA GCTATGATAA AC                                                    22

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:

51

```
            (A)  LENGTH: 21 base pairs
            (B)  TYPE: nucleic acid
            (C)  STRANDEDNESS: single
            (D)  TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCTAGCTGAA GCAAAACAGT G                                          21

(2) INFORMATION FOR SEQ ID NO:3:

      (i) SEQUENCE CHARACTERISTICS:
            (A)  LENGTH: 19 base pairs
            (B)  TYPE: nucleic acid
            (C)  STRANDEDNESS: single
            (D)  TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATTGGTCCGC CCCTGAGGT                                             19

(2) INFORMATION FOR SEQ ID NO:4:

      (i) SEQUENCE CHARACTERISTICS:
            (A)  LENGTH: 20 base pairs
            (B)  TYPE: nucleic acid
            (C)  STRANDEDNESS: single
            (D)  TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGATGCAGGA ATCTGGCTCT                                            20

(2) INFORMATION FOR SEQ ID NO:5:

      (i) SEQUENCE CHARACTERISTICS:
            (A)  LENGTH: 20 base pairs
            (B)  TYPE: nucleic acid
            (C)  STRANDEDNESS: single
            (D)  TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTTTGGATCC ACCATGGCTA                                            20

(2) INFORMATION FOR SEQ ID NO:6:

      (i) SEQUENCE CHARACTERISTICS:
            (A)  LENGTH: 24 base pairs
            (B)  TYPE: nucleic acid
            (C)  STRANDEDNESS: single
            (D)  TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTTTGCCGGC ACTGCCCTCT TGAA                                       24

(2) INFORMATION FOR SEQ ID NO:7:

      (i) SEQUENCE CHARACTERISTICS:
            (A)  LENGTH: 24 base pairs
            (B)  TYPE: nucleic acid
```

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```
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
```

TTTTCAGCTG GACAATGGAT TGGC                                                                  24

(2) INFORMATION FOR SEQ ID NO:8:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
```

TTTTGCTAGC TGGCGAATCC                                                                        20

(2) INFORMATION FOR SEQ ID NO:9:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
```

TTTTGGATCC GTGTCCCATA GTGTTTCCAA                                                             30

(2) INFORMATION FOR SEQ ID NO:10:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
```

TTTTGGATCC GCAGTCGTGG CCAGTACC                                                               28

(2) INFORMATION FOR SEQ ID NO:11:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
```

CTAGTCCTAG GA                                                                                12

(2) INFORMATION FOR SEQ ID NO:12:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
```

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTTTGAGCAC AGAGCCTCGC CT                                    22

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTTTGGATCC GGTGAGCTGC GAGAATAGCC                            30

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 76 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGGCCCCCAG CCCCAGCCCT CCCATTGGTG GAGGCCCTTT TGGAGGCACC CTAGGGCCAG    60

GAAACTTTTG CCGTAT                                          76

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 69 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAATAGGGCA GATCCGGGCT TTATTATTTT AGCACCACGG CCGCCGAGAC CGCGTCCGCC    60

CCGCGAGCA                                                 69

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 86 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGCCCTATTT ATACGGCAAA AGTTTCCTGG CCCTAGGGTG CCTCCAAAAG GGCCTCCACC    60

AATGGGAGGG CTGGGGCTGG GGGCCC                              86

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:

54

```
            (A) LENGTH: 55 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGCGGGGCGG ACGCGGTCTC GGCGGCCGTG GTGCTAAAAT AATAAAGCCC GGATC            55

(2) INFORMATION FOR SEQ ID NO:18:

            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 1343 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCGCGGGAAA TTTATGCTGT CCGGTCACCG TGACAATGCA GCTGAGGAAC CCAGAACTAC         60

ATCTGGGCTG CGCGCTTGCG CTTCGCTTCC TGGCCCTCGT TTCCTGGGAC ATCCCTGGGG        120

CTAGAGCACT GGACAATGGA TTGGCAAGGA CGCCTACCAT GGGCTGGCTG CACTGGGAGC        180

GCTTCATGTG CAACCTTGAC TGCCAGGAAG AGCCAGATTC CTGCATCAGT GAGAAGCTCT        240

TCATGGAGAT GGCAGAGCTC ATGGTCTCAG AAGGCTGGAA GGATGCAGGT TATGAGTACC        300

TCTGCATTGA TGACTGTTGG ATGGCTCCCC AAAGAGATTC AGAAGGCAGA CTTCAGGCAG        360

ACCCTCAGCG CTTTCCTCAT GGGATTCGCC AGCTAGCTAA TTATGTTCAC AGCAAAGGAC        420

TGAAGCTAGG GATTTATGCA GATGTTGGAA ATAAAACCTG CGCAGGCTTC CCTGGGAGTT        480

TTGGATACTA CGACATTGAT GCCCAGACCT TTGCTGACTG GGGAGTAGAT CTGCTAAAAT        540

TTGATGGTTG TTACTGTGAC AGTTTGGAAA ATTTGGCAGA TGGTTATAAG CACATGTCCT        600

TGGCCCTGAA TAGGACTGGC AGAAGCATTG TGTACTCCTG TGAGTGGCCT CTTTATATGT        660

GGCCCTTTCA AAAGCCCAAT TATACAGAAA TCCGACAGTA CTGCAATCAC TGGCGAAATT        720

TTGCTGACAT TGATGATTCC TGGAAAAGTA TAAAGAGTAT CTTGGACTGG ACATCTTTTA        780

ACCAGGAGAG AATTGTTGAT GTTGCTGGAC CAGGGGGTTG GAATGACCCA GATATGTTAG        840

TGATTGGCAA CTTTGGCCTC AGCTGGAATC AGCAAGTAAC TCAGATGGCC CTCTGGGCTA        900

TCATGGCTGC TCCTTTATTC ATGTCTAATG ACCTCCGACA CATCAGCCCT CAAGCCAAAG        960

CTCTCCTTCA GGATAAGGAC GTAATTGCCA TCAATCAGGA CCCCTTGGGC AAGCAAGGGT       1020

ACCAGCTTAG ACAGGGAGAC AACTTTGAAG TGTGGGAACG ACCTCTCTCA GGCTTAGCCT       1080

GGGCTGTAGC TATGATAAAC CGGCAGGAGA TTGGTGGACC TCGCTCTTAT ACCATCGCAG       1140

TTGCTTCCCT GGGTAAAGGA GTGGCCTGTA ATCCTGCCTG CTTCATCACA CAGCTCCTCC       1200

CTGTGAAAAG GAAGCTAGGG TTCTATGAAT GGACTTCAAG GTTAAGAAGT CACATAAATC       1260

CCACAGGCAC TGTTTTGCTT CAGCTAGAAA ATACAATGCA GATGTCATTA AAAGACTTAC       1320
```

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TTTAAAAAAA AAAAAAACTC GAG                                                1343

(2) INFORMATION FOR SEQ ID NO:19:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 210 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTGGGCTGTA GCTATGATAA ACCGGCAGGA GATTGGTGGA CCTCGCTCTT ATACCATCGC      60

AGTTGCTTCC CTGGGTAAAG GAGTGGCCTG TAATCCTGCC TGCTTCATCA CACAGCTCCT      120

CCCTGTGAAA AGGAAGCTAG GGTTCTATGA ATGGACTTCA AGGTTAAGAA GTCACATAAA      180

TCCCACAGGC ACTGTTTTGC TTCAGCTAGA                                        210

(2) INFORMATION FOR SEQ ID NO:20:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 268 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATTGGTCCGC CCCTGAGGTT AATCTTAAAA GCCCAGGTTA CCCGCGGAAA TTTATGCTGT      60

CCGGTCACCG TGACAATGCA GCTGAGGAAC CCAGAACTAC ATCTGGGCTG CGCGCTTGCG      120

CTTCGCTTCC TGGCCCTCGT TTCCTGGGAC ATCCCTGGGG CTAGAGCACT GGACAATGGA      180

TTGGCAAGGA CGCCTACCAT GGGCTGGCTG CACTGGGAGC GCTTCATGTG CAACCTTGAC      240

TGCCAGGAAG AGCCAGATTC CTGCATCA                                          268

(2) INFORMATION FOR SEQ ID NO:21:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 26 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

    Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
    1               5                   10                  15

    Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala
                20                  25

(2) INFORMATION FOR SEQ ID NO:22:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 78 base pairs

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```
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGGCTACAG GCTCCCGGAC GTCCCTGCTC CTGGCTTTTG GCCTGCTCTG CCTGCCCTGG        60

CTTCAAGAGG GCAGTGCC                                        .            78

(2) INFORMATION FOR SEQ ID NO:23:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTTTGGATCC CTCGAGGACA TTGATTATTG ACTAG                                  35

(2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TTTTGGATCC CGTGTCAAGG ACGGTGAC                                          28

(2) INFORMATION FOR SEQ ID NO:25:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1197 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTGGACAATG GATTGGCAAG GACGCCTACC ATGGGCTGGC TGCACTGGGA GCGCTTCATG        60

TGCAACCTTG ACTGCCAGGA AGAGCCAGAT TCCTGCATCA GTGAGAAGCT CTTCATGGAG       120

ATGGCAGAGC TCATGGTCTC AGAAGGCTGG AAGGATGCAG GTTATGAGTA CCTCTGCATT       180

GATGACTGTT GGATGGCTCC CCAAAGAGAT TCAGAAGGCA GACTTCAGGC AGACCCTCAG       240

CGCTTTCCTC ATGGGATTCG CCAGCTAGCT AATTATGTTC ACAGCAAAGG ACTGAAGCTA       300

GGGATTTATG CAGATGTTGG AAATAAAACC TGCGCAGGCT TCCCTGGGAG TTTTGGATAC       360

TACGACATTG ATGCCCAGAC CTTTGCTGAC TGGGGAGTAG ATCTGCTAAA ATTTGATGGT       420

TGTTACTGTG ACAGTTTGGA AAATTTGGCA GATGGTTATA AGCACATGTC CTTGGCCCTG       480
```

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```
AATAGGACTG GCAGAAGCAT TGTGTACTCC TGTGAGTGGC CTCTTTATAT GTGGCCCTTT      540

CAAAAGCCCA ATTATACAGA AATCCGACAG TACTGCAATC ACTGGCGAAA TTTTGCTGAC      600

ATTGATGATT CCTGGAAAAG TATAAAGAGT ATCTTGGACT GGACATCTTT TAACCAGGAG      660

AGAATTGTTG ATGTTGCTGG ACCAGGGGGT TGGAATGACC CAGATATGTT AGTGATTGGC      720

AACTTTGGCC TCAGCTGGAA TCAGCAAGTA ACTCAGATGG CCCTCTGGGC TATCATGGCT      780

GCTCCTTTAT TCATGTCTAA TGACCTCCGA CACATCAGCC CTCAAGCCAA AGCTCTCCTT      840

CAGGATAAGG ACGTAATTGC CATCAATCAG GACCCCTTGG GCAAGCAAGG GTACCAGCTT      900

AGACAGGGAG ACAACTTTGA AGTGTGGGAA CGACCTCTCT CAGGCTTAGC CTGGGCTGTA      960

GCTATGATAA ACCGGCAGGA GATTGGTGGA CCTCGCTCTT ATACCATCGC AGTTGCTTCC     1020

CTGGGTAAAG GAGTGGCCTG TAATCCTGCC TGCTTCATCA CACAGCTCCT CCCTGTGAAA     1080

AGGAAGCTAG GGTTCTATGA ATGGACTTCA AGGTTAAGAA GTCACATAAA TCCCACAGGC     1140

ACTGTTTTGC TTCAGCTAGA AAATACAATG CAGATGTCAT TAAAAGACTT ACTTTAA        1197
```

(2) INFORMATION FOR SEQ ID NO:26:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 398 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
Leu Asp Asn Gly Leu Ala Arg Thr Pro Thr Met Gly Trp Leu His Trp
1               5               10              15

Glu Arg Phe Met Cys Asn Leu Asp Cys Gln Glu Glu Pro Asp Ser Cys
            20              25              30

Ile Ser Glu Lys Leu Phe Met Glu Met Ala Glu Leu Met Val Ser Glu
            35              40              45

Gly Trp Lys Asp Ala Gly Tyr Glu Tyr Leu Cys Ile Asp Asp Cys Trp
        50              55              60

Met Ala Pro Gln Arg Asp Ser Glu Gly Arg Leu Gln Ala Asp Pro Gln
65              70              75              80

Arg Phe Pro His Gly Ile Arg Gln Leu Ala Asn Tyr Val His Ser Lys
            85              90              95

Gly Leu Lys Leu Gly Ile Tyr Ala Asp Val Gly Asn Lys Thr Cys Ala
            100             105             110

Gly Phe Pro Gly Ser Phe Gly Tyr Tyr Asp Ile Asp Ala Gln Thr Phe
            115             120             125

Ala Asp Trp Gly Val Asp Leu Leu Lys Phe Asp Gly Cys Tyr Cys Asp
            130             135             140
```

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```
Ser Leu Glu Asn Leu Ala Asp Gly Tyr Lys His Met Ser Leu Ala Leu
145                 150                 155                 160

Asn Arg Thr Gly Arg Ser Ile Val Tyr Ser Cys Glu Trp Pro Leu Tyr
                165                 170                 175

Met Trp Pro Phe Gln Lys Pro Asn Tyr Thr Glu Ile Arg Gln Tyr Cys
            180                 185                 190

Asn His Trp Arg Asn Phe Ala Asp Ile Asp Asp Ser Trp Lys Ser Ile
        195                 200                 205

Lys Ser Ile Leu Asp Trp Thr Ser Phe Asn Gln Glu Arg Ile Val Asp
        210                 215                 220

Val Ala Gly Pro Gly Gly Trp Asn Asp Pro Asp Met Leu Val Ile Gly
225                 230                 235                 240

Asn Phe Gly Leu Ser Trp Asn Gln Gln Val Thr Gln Met Ala Leu Trp
                245                 250                 255

Ala Ile Met Ala Ala Pro Leu Phe Met Ser Asn Asp Leu Arg His Ile
            260                 265                 270

Ser Pro Gln Ala Lys Ala Leu Leu Gln Asp Lys Asp Val Ile Ala Ile
        275                 280                 285

Asn Gln Asp Pro Leu Gly Lys Gln Gly Tyr Gln Leu Arg Gln Gly Asp
    290                 295                 300

Asn Phe Glu Val Trp Glu Arg Pro Leu Ser Gly Leu Ala Trp Ala Val
305                 310                 315                 320

Ala Met Ile Asn Arg Gln Glu Ile Gly Gly Pro Arg Ser Tyr Thr Ile
                325                 330                 335

Ala Val Ala Ser Leu Gly Lys Gly Val Ala Cys Asn Pro Ala Cys Phe
            340                 345                 350

Ile Thr Gln Leu Leu Pro Val Lys Arg Lys Leu Gly Phe Tyr Glu Trp
        355                 360                 365

Thr Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr Val Leu Leu
    370                 375                 380

Gln Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp Leu Leu
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:27:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 338 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
ATGGCTACAG GTAAGCGCCC CTAAAATCCC TTTGGGCACA ATGTGTCCTG AGGGGAGAGG        60

CAGCGACCTG TAGATGGGAC GGGGGCACTA ACCCTCAGGT TTGGGGCTTC TGAATGTGAG       120
```

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TATCGCCATG TAAGCCCAGT ATTTGGCCAA TCTCAGAAAG CTCCTGGTCC CTGGAGGGAT          180

GGAGAGAGAA AAACAAACAG CTCCTGGAGC AGGGAGAGTG CTGGCCTCTT GCTCTCCGGC          240

TCCCTCTGTT GCCCTCTGGT TTCTCCCCAG GCTCCCGGAC GTCCCTGCTC CTGGCTTTTG          300

GCCTGCTCTG CCTGCCCTGG CTTCAAGAGG GCAGTGCC                                  338

(2) INFORMATION FOR SEQ ID NO:28:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

    Leu Asp Asn Gly Leu Ala Arg Thr Pro Thr
    1               5                   10

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What is claimed is:

1. A method of treatment comprising identifying a patient suspected of having an α-galactosidase A deficiency, and introducing into the patient a human cell

5 genetically modified to overexpress and secrete human α-gal A.

2. The method of claim 1, wherein the cell has been transfected *in vitro* with a DNA molecule having a coding sequence encoding a polypeptide comprising human

10 α-gal A (SEQ ID NO:26).

3. The method of claim 2, wherein the polypeptide further comprises an heterologous signal peptide.

4. The method of claim 3, wherein the heterologous signal peptide is the human growth hormone

15 (hGH) signal peptide (SEQ ID NO:21).

5. The method of claim 3, wherein the DNA molecule comprises an intron within the sequence encoding the signal peptide.

6. The method of claim 3, wherein the DNA

20 molecule comprises an untranslated sequence of at least 6 nucleotides lying immediately 3' to the termination codon of the coding sequence, said untranslated sequence comprising a polyadenylation site.

7. A DNA molecule comprising

25 a first sequence encoding the hGH signal peptide (SEQ ID NO:21), said first sequence comprising an intron; and,

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linked to the 3' end of the first sequence, a
second sequence encoding a polypeptide comprising human
α-gal A.

8.   The DNA molecule of claim 7, further
5  comprising a 3' untranslated sequence comprising a
polyadenylation site.

9.   An expression construct comprising the DNA
molecule of claim 7 and suitable for expression in a
human cell.

10        10.   A cultured human cell containing a DNA
molecule that (a) encodes a polypeptide comprising human
α-gal A linked to an heterologous signal peptide, and
(b) permits expression of the polypeptide in the cell.

11.   The cell of claim 10, said cell being a
15  fibroblast.

12.   The cell of claim 10, wherein said cell is
selected from the group consisting of an epithelial cell,
an endothelial cell, a bone marrow cell, a glial cell, a
hepatocyte, a keratinocyte, a myocyte, a neuron, or a
20  precursor of these cell types.

13.   The cell of claim 10, wherein said signal
peptide is the hGH signal peptide (SEQ ID NO:21).

14.   A clonal cell strain consisting essentially
of a plurality of cultured human cells transfected with a
25  DNA molecule that (a) encodes a polypeptide comprising
human α-gal A linked to an heterologous signal peptide,
and (b) permits overexpression of the polypeptide in the
cells.

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15.    The clonal cell strain of claim 14, said
cells being fibroblasts.


16.    A clonal cell line consisting essentially of
a plurality of immortalized human cells transfected with
5 a DNA molecule that (a) encodes a polypeptide comprising
human α-gal A linked to an heterologous signal peptide,
and (b) permits overexpression of the polypeptide in the
cells.


17.    The clonal cell line of claim 16, wherein
10 said cell is selected from the group consisting of a
Bowes melanoma cell, a Daudi cell, a HeLa cell, an HL-60
cell, an HT1080 cell, a Jurkat cell, a KB carcinoma cell,
a K-562 leukemia cell, an MCF-7 breast cancer cell, a
MOLT-4 cell, a Namalwa cell, a Raji cell, an RPMI 8226
15 cell, a U-937 cell, a WI-38VA13 (subline 2R4) cell, and a
2780AD ovarian carcinoma cell.


18.    A protein comprising (a) the hGH signal
peptide (SEQ ID NO:21) linked by a peptide bond to (b)
human α-gal A.


20         19.    A process for making glycosylated human
α-gal A, comprising
         culturing the cell of claim 11 under conditions
permitting expression of human α-gal A from said DNA
molecule and secretion of glycosylated human α-gal A into
25 the culture medium of the cell; and
         isolating glycosylated human α-gal A from the
culture medium.


20.    Purified glycosylated human α-gal A made by
the process of claim 19.

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21. A process for purifying human α-gal A from a
sample, comprising a first chromatography step wherein
the sample is passed over a hydrophobic interaction
resin.

5          22. The process of claim 21, wherein the
functional moiety on the resin comprises a butyl group.

23. A process for making glycosylated human
α-gal A, comprising
culturing the cell of claim 16 under conditions
10  permitting expression of human α-gal A from said DNA
molecule and secretion of glycosylated human α-gal A into
the culture medium of the cell; and
isolating glycosylated human α-gal A from the
culture medium.

15         24. The process of claim 21, further comprising
the step of passing the sample over a second resin
selected from the group consisting of an immobilized
heparin resin, hydroxyapatite, an anion exchange resin,
and a size exclusion resin.

20         25. A method of treatment comprising
identifying a patient suspected of having an
α-gal A deficiency, and
administering to the patient the purified
glycosylated human α-gal A of claim 20.

25         26. A therapeutic composition comprising
pharmaceutically acceptable purified human α-gal A having
N-linked mannose-6-phosphate in a pharmaceutically
acceptable carrier.

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27. A therapeutic composition comprising the purified human α-gal A of claim 20 and a pharmaceutically acceptable excipient.

28. The therapeutic composition of claim 27,
5 formulated at pH 6.5 or lower.

29. The therapeutic composition of claim 28, wherein the pharmaceutically acceptable excipient is human serum albumin.

30. A DNA molecule encoding a protein comprising
10 the signal peptide of hGH (SEQ ID NO:21) linked to a polypeptide other than hGH.

31. The DNA molecule of claim 30, wherein the DNA molecule comprises an intron within the sequence encoding the signal peptide.

15       32. A cultured human cell containing a DNA molecule that (a) encodes a polypeptide comprising human α-gal A, and (b) permits overexpression of the polypeptide in the cell.

33. A process for making glycosylated human
20 α-gal A, comprising culturing a human cell containing a DNA molecule that encodes a polypeptide comprising human α-gal A under conditions permitting expression of human α-gal A from said DNA molecule.

34. The therapeutic composition of claim 26
25 further comprising a pharmaceutically acceptable excipient.

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35.   A process for purifying human α-gal A from a
sample comprising passing said sample over a hydrophobic
interaction resin comprising a butyl group as a
functional moiety.

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CTGGGCTGTAGCTATGATAAACCGGCAGGA

GATTGGTGGACCTCGCTCTTATACCATCGCA

GTTGCTTCCCTGGGTAAAGGAGTGGCCTGTA

ATCCTGCCTGCTTCATCACACAGCTCCTCCCT

GTGAAAAGGAAGCTAGGGTTCTATGAATGGA

CTTCAAGGTTAAGAAGTCACATAAATCCCAC

AGGCACTGTTTTGCTTCAGCTAGA

# FIG. 1

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ATTGGTCCGCCCCTGAGGTTAATCTTAAAAG

SacII
CCCAGGTTACCCGCGGAAATTTATGCTGTC

CGGTCACCGTGACAATGCAGCTGAGGAACC

CAGAACTACATCTGGGCTGCGCGCTTGCGCT

TCGCTTCCTGGCCCTCGTTTCCTGGGACATC

CCTGGGGCTAGAGCACTGGACAATGGATTG

NcoI
GCAAGGACGCCTACCATGGGCTGGCTGCAC

TGGGAGCGCTTCATGTGCAACCTTGACTGCC

AGGAAGAGCCAGATTCCTGCATCA

# FIG. 2

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```
   1 CCGCGGGAAA TTTATGCTGT CCGGTCACCG TGACAATGCA GCTGAGGAAC CCAGAACTAC
  61 ATCTGGGCTG CGCGCTTGCG CTTCGCTTCC TGGCCCTCGT TTCCTGGGAC ATCCCTGGGG
 121 CTAGAGCACT GGACAATGGA TTGGCAAGGA CGCCTACCAT GGGCTGGCTG CACTGGGAGC
 181 GCTTCATGTG CAACCTTGAC TGCCAGGAAG AGCCAGATTC CTGCATCAGT GAGAAGCTCT
 241 TCATGGAGAT GGCAGAGCTC ATGGTCTCAG AAGGCTGGAA GGATGCAGGT TATGAGTACC
 301 TCTGCATTGA TGACTGTTGG ATGGCTCCCC AAAGAGATTC AGAAGGCAGA CTTCAGGCAG
 361 ACCCTCAGCG CTTTCCTCAT GGGATTCGCC AGCTAGCTAA TTATGTTCAC AGCAAAGGAC
 421 TGAAGCTAGG GATTTATGCA GATGTTGGAA ATAAAACCTG CGCAGGCTTC CCTGGGAGTT
 481 TTGGATACTA CGACATTGAT GCCCAGACCT TTGCTGACTG GGGAGTAGAT CTGCTAAAAT
 541 TTGATGGTTG TTACTGTGAC AGTTTGGAAA ATTTGGCAGA TGGTTATAAG CACATGTCCT
 601 TGGCCCTGAA TAGGACTGGC AGAAGCATTG TGTACTCCTG TGAGTGGCCT CTTTATATGT
 661 GGCCCTTTCA AAAGCCCAAT TATACAGAAA TCCGACAGTA CTGCAATCAC TGGCGAAATT
 721 TTGCTGACAT TGATGATTCC TGGAAAAGTA TAAAGAGTAT CTTGGACTGG ACATCTTTTA
 781 ACCAGGAGAG AATTGTTGAT GTTGCTGGAC CAGGGGGTTG GAATGACCCA GATATGTTAG
 841 TGATTGGCAA CTTTGGCCTC AGCTGGAATC AGCAAGTAAC TCAGATGGCC CTCTGGGCTA
 901 TCATGGCTGC TCCTTTATTC ATGTCTAATG ACCTCCGACA CATCAGCCCT CAAGCCAAAG
 961 CTCTCCTTCA GGATAAGGAC GTAATTGCCA TCAATCAGGA CCCCTTGGGC AAGCAAGGGT
1021 ACCAGCTTAG ACAGGGAGAC AACTTTGAAG TGTGGGAACG ACCTCTCTCA GGCTTAGCCT
1081 GGGCTGTAGC TATGATAAAC CGGCAGGAGA TTGGTGGACC TCGCTCTTAT ACCATCGCAG
1141 TTGCTTCCCT GGGTAAAGGA GTGGCCTGTA ATCCTGCCTG CTTCATCACA CAGCTCCTCC
1201 CTGTGAAAAG GAAGCTAGGG TTCTATGAAT GGACTTCAAG GTTAAGAAGT CACATAAATC
1261 CCACAGGCAC TGTTTTGCTT CAGCTAGAAA ATACAATGCA GATGTCATTA AAAGACTTAC
1321 TTTAAAAAAA AAAAAAACTC GAG
```
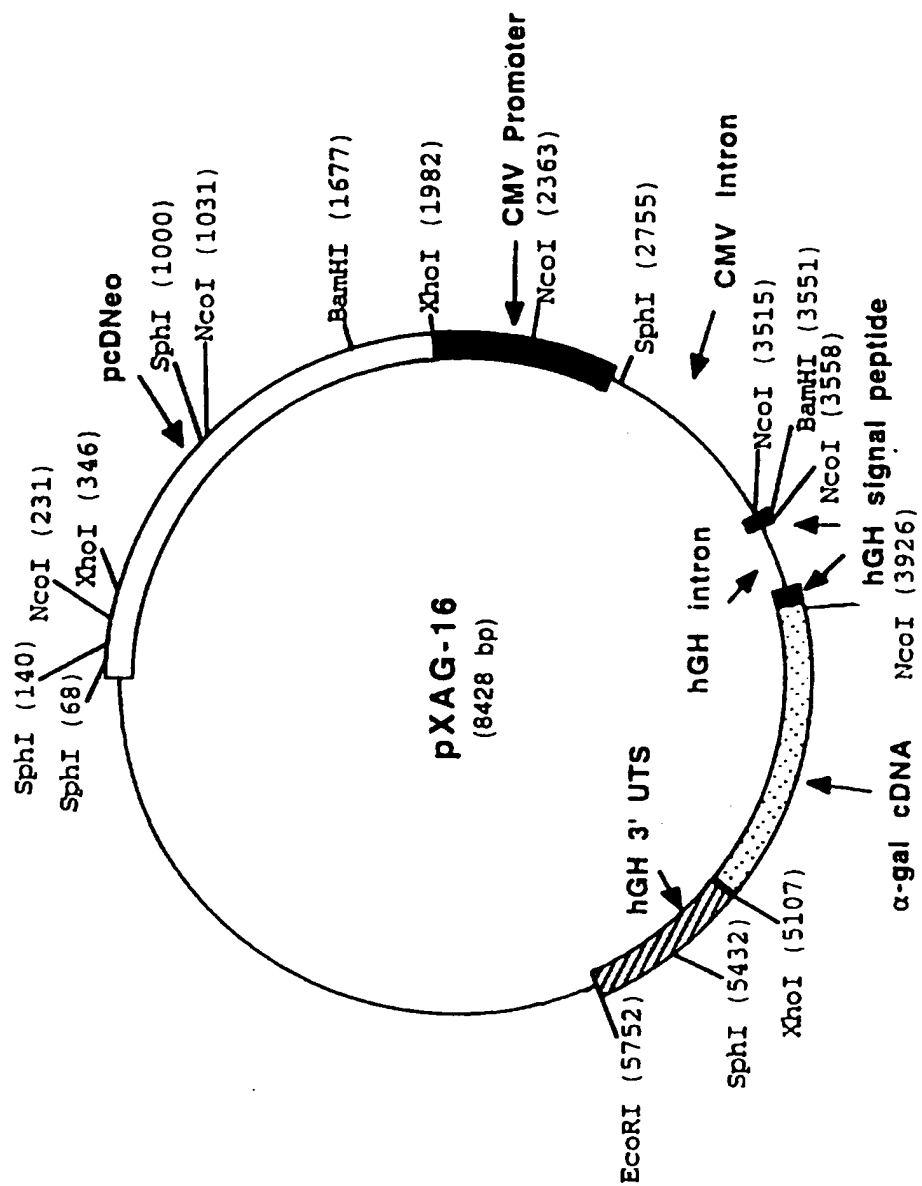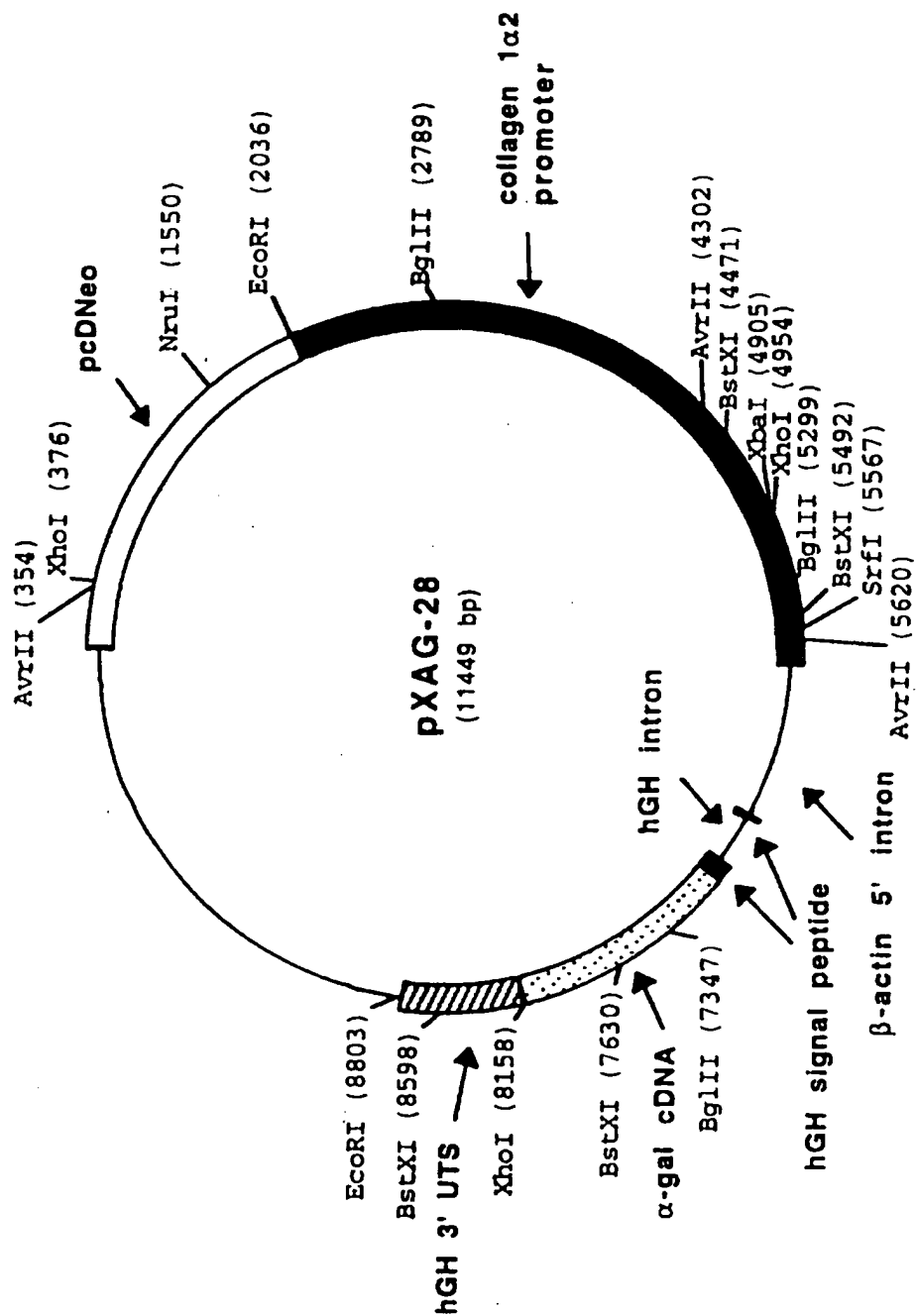
**FIG. 3**

**FIG. 4**

pXAG-16
(8428 bp)

SphI (68)
SphI (140)
NcoI (231)
XhoI (346)
pcDNeo
SphI (1000)
NcoI (1031)
BamHI (1677)
XhoI (1982)
CMV Promoter
NcoI (2363)
SphI (2755)
CMV Intron
NcoI (3515)
BamHI (3551)
NcoI (3558)
hGH signal peptide
NcoI (3926)
hGH intron
α-gal cDNA
XhoI (5107)
SphI (5432)
hGH 3' UTS
EcoRI (5752)

FIG. 5
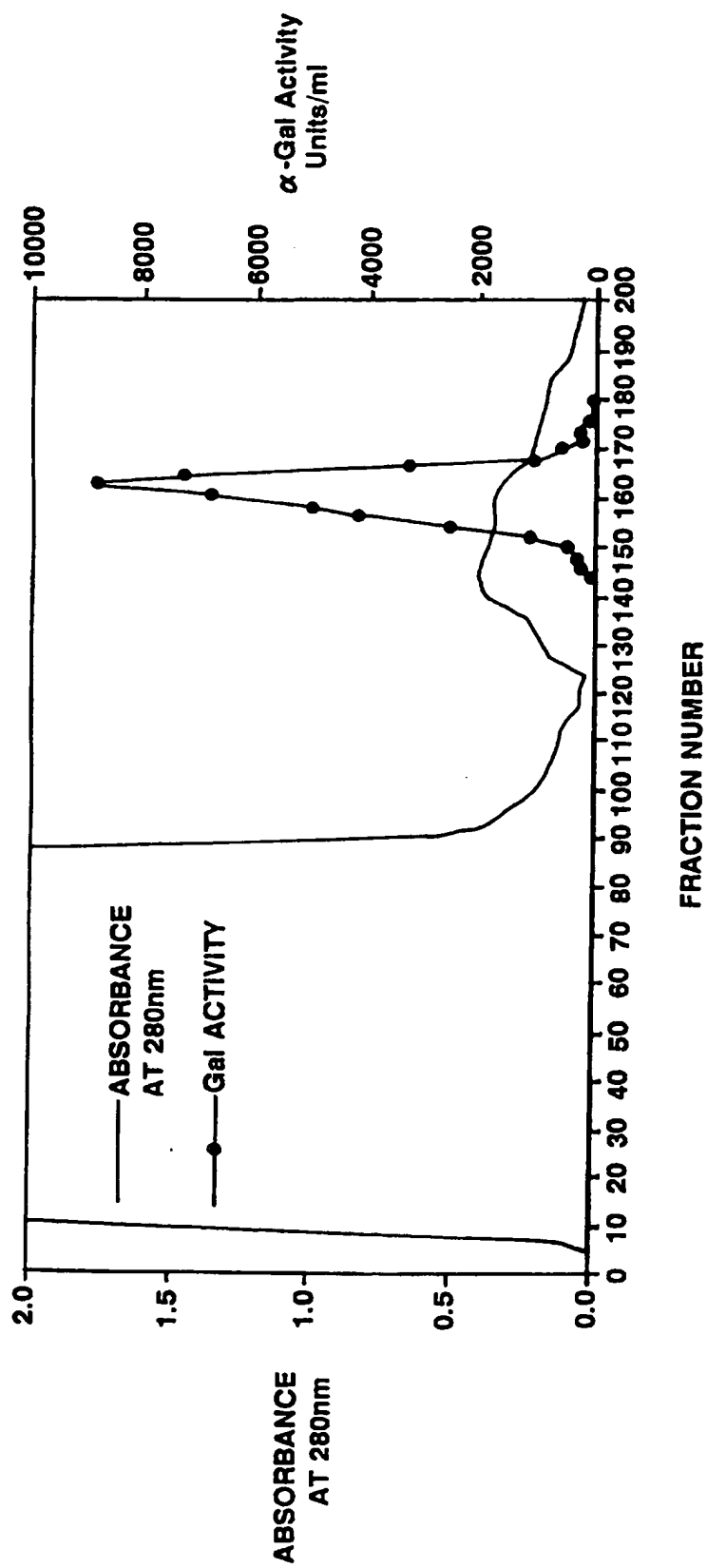
FIG. 6
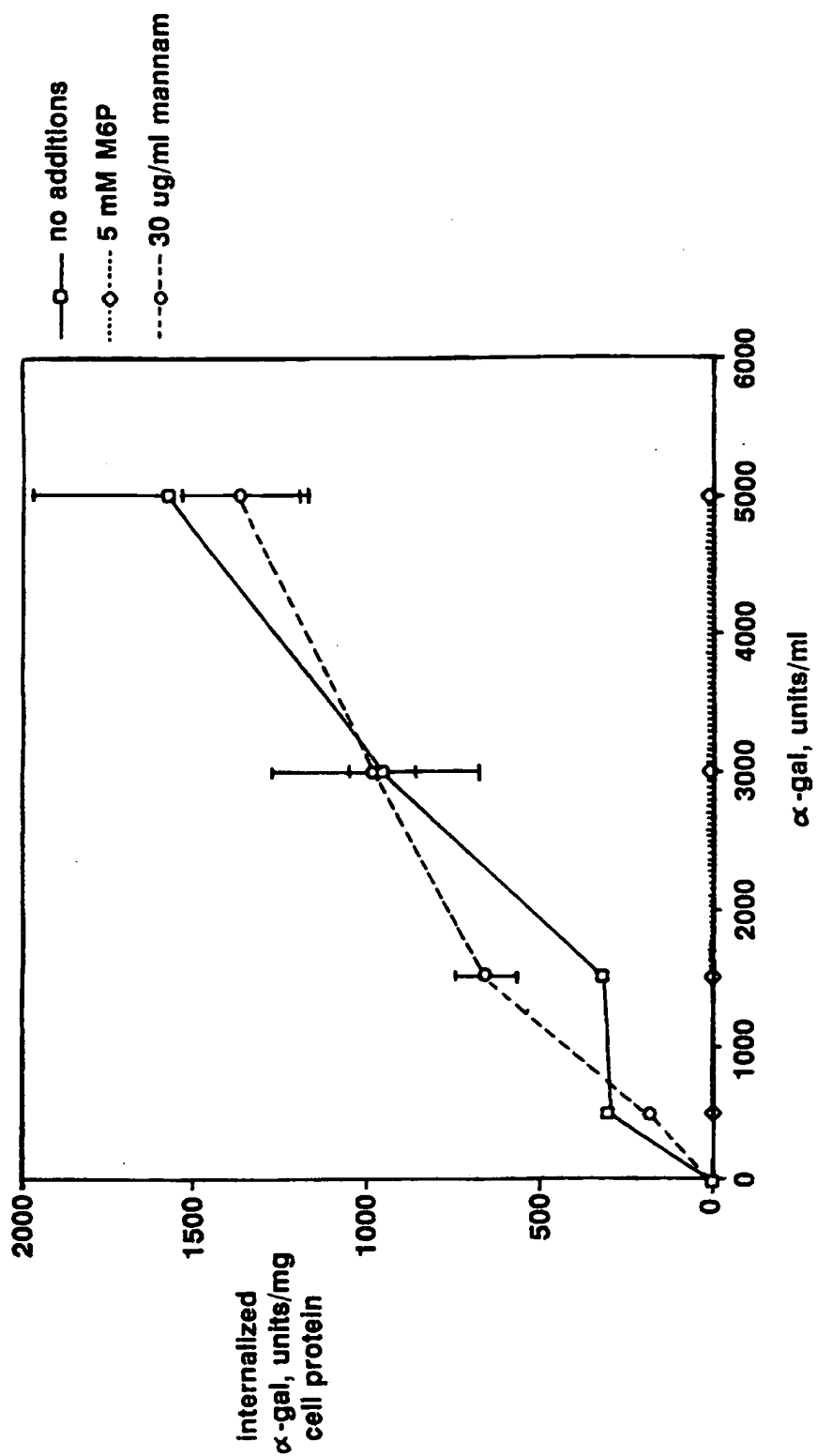
FIG. 7
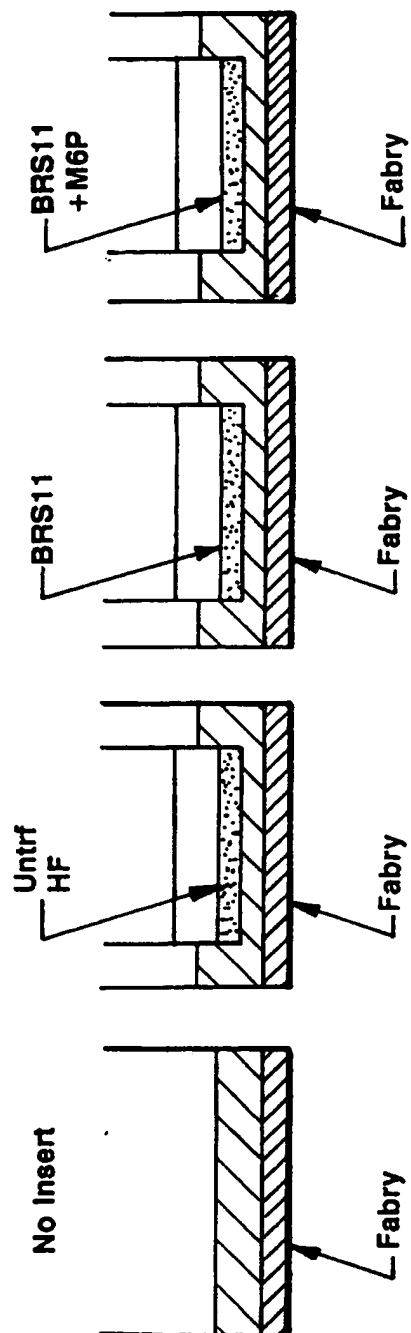
**FIG. 8**

| Leu | Asp | Asn | Gly | Leu | Ala | Arg | Thr | Pro | Thr | Met | Gly |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Trp | Leu | His | Trp | Glu | Arg | Phe | Met | Cys | Asn | Leu | Asp |
| Cys | Gln | Glu | Glu | Pro | Asp | Ser | Cys | Ile | Ser | Glu | Lys |
| Leu | Phe | Met | Glu | Met | Ala | Glu | Leu | Met | Val | Ser | Glu |
| Gly | Trp | Lys | Asp | Ala | Gly | Tyr | Glu | Tyr | Leu | Cys | Ile |
| Asp | Asp | Cys | Trp | Met | Ala | Pro | Gln | Arg | Asp | Ser | Glu |
| Gly | Arg | Leu | Gln | Ala | Asp | Pro | Gln | Arg | Phe | Pro | His |
| Gly | Ile | Arg | Gln | Leu | Ala | Asn | Tyr | Val | His | Ser | Lys |
| Gly | Leu | Lys | Leu | Gly | Ile | Tyr | Ala | Asp | Val | Gly | Asn |
| Lys | Thr | Cys | Ala | Gly | Phe | Pro | Gly | Ser | Phe | Gly | Tyr |
| Tyr | Asp | Ile | Asp | Ala | Gln | Thr | Phe | Ala | Asp | Trp | Gly |
| Val | Asp | Leu | Leu | Lys | Phe | Asp | Gly | Cys | Tyr | Cys | Asp |
| Ser | Leu | Glu | Asn | Leu | Ala | Asp | Gly | Tyr | Lys | His | Met |
| Ser | Leu | Ala | Leu | Asn | Arg | Thr | Gly | Arg | Ser | Ile | Val |
| Tyr | Ser | Cys | Glu | Trp | Pro | Leu | Tyr | Met | Trp | Pro | Phe |
| Gln | Lys | Pro | Asn | Tyr | Thr | Glu | Ile | Arg | Gln | Tyr | Cys |
| Asn | His | Trp | Arg | Asn | Phe | Ala | Asp | Ile | Asp | Asp | Ser |
| Trp | Lys | Ser | Ile | Lys | Ser | Ile | Leu | Asp | Trp | Thr | Ser |
| Phe | Asn | Gln | Glu | Arg | Ile | Val | Asp | Val | Ala | Gly | Pro |
| Gly | Gly | Trp | Asn | Asp | Pro | Asp | Met | Leu | Val | Ile | Gly |
| Asn | Phe | Gly | Leu | Ser | Trp | Asn | Gln | Gln | Val | Thr | Gln |
| Met | Ala | Leu | Trp | Ala | Ile | Met | Ala | Ala | Pro | Leu | Phe |
| Met | Ser | Asn | Asp | Leu | Arg | His | Ile | Ser | Pro | Gln | Ala |
| Lys | Ala | Leu | Leu | Gln | Asp | Lys | Asp | Val | Ile | Ala | Ile |
| Asn | Gln | Asp | Pro | Leu | Gly | Lys | Gln | Gly | Tyr | Gln | Leu |
| Arg | Gln | Gly | Asp | Asn | Phe | Glu | Val | Trp | Glu | Arg | Pro |
| Leu | Ser | Gly | Leu | Ala | Trp | Ala | Val | Ala | Met | Ile | Asn |
| Arg | Gln | Glu | Ile | Gly | Gly | Pro | Arg | Ser | Tyr | Thr | Ile |
| Ala | Val | Ala | Ser | Leu | Gly | Lys | Gly | Val | Ala | Cys | Asn |
| Pro | Ala | Cys | Phe | Ile | Thr | Gln | Leu | Leu | Pro | Val | Lys |
| Arg | Lys | Leu | Gly | Phe | Tyr | Glu | Trp | Thr | Ser | Arg | Leu |
| Arg | Ser | His | Ile | Asn | Pro | Thr | Gly | Thr | Val | Leu | Leu |
| Gln | Leu | Glu | Asn | Thr | Met | Gln | Met | Ser | Leu | Lys | Asp |
| Leu | Leu |     |     |     |     |     |     |     |     |     |     |

**FIG. 9**

ATGGCTACAG  GTAAGCGCCC  CTAAAATCCC  TTTGGGCACA

ATGTGTCCTG  AGGGGAGAGG  CAGCGACCTG  TAGATGGGAC

GGGGGCACTA  ACCCTCAGGT  TTGGGGCTTC  TGAATGTGAG

TATCGCCATG  TAAGCCCAGT  ATTTGGCCAA  TCTCAGAAAG

CTCCTGGTCC  CTGGAGGGAT  GGAGAGAGAA  AAACAAACAG

CTCCTGGAGC  AGGGAGAGTG  CTGGCCTCTT  GCTCTCCGGC

TCCCTCTGTT  GCCCTCTGGT  TTCTCCCCAG  GCTCCCGGAC

GTCCCTGCTC  CTGGCTTTTG  GCCTGCTCTG  CCTGCCCTGG

CTTCAAGAGG  GCAGTGCC

# FIG. 10

ATGGCTACAG  GCTCCCGGAC  GTCCCTGCTC  CTGGCTTTTG

GCCTGCTCTG  CCTGCCCTGG  CTTCAAGAGG  GCAGTGCC

# FIG. 11

Met  Ala  Thr  Gly  Ser  Arg  Thr  Ser  Leu  Leu  Leu  Ala
Phe  Gly  Leu  Leu  Cys  Leu  Pro  Trp  Leu  Gln  Glu  Gly
Ser  Ala

# FIG. 12

```
   1  CTGGACAATG GATTGGCAAG GACGCCTACC ATGGGCTGGC TGCACTGGGA GCGCTTCATG
  61  TGCAACCTTG ACTGCCAGGA AGAGCCAGAT TCCTGCATCA GTGAGAAGCT CTTCATGGAG
 121  ATGGCAGAGC TCATGGTCTC AGAAGGCTGG AAGGATGCAG GTTATGAGTA CCTCTGCATT
 181  GATGACTGTT GGATGGCTCC CCAAAGAGAT TCAGAAGGCA GACTTCAGGC AGACCCTCAG
 241  CGCTTCCTC  ATGGGATTCG CCAGCTAGCT AATTATGTTC ACAGCAAAGG ACTGAAGCTA
 301  GGGATTTATG CAGATGTTGG AAATAAAACC TGGCCAGGCT TCCCTGGGAG TTTTGGATAC
 361  TACGACATTG ATGCCCAGAC CTTTGCTGAC TGGGGAGTAG ATCTGCTAAA ATTTGATGGT
 421  TGTTACTGTG ACAGTTTGGA AAATTTGGCA GATGGTTATA AGCACATGTC CTTGGCCCTG
 481  AATAGGACTG GCAGAAGCAT ATTATACAGA TGTGTACTCC TGTGAGTGGC CTCTTTATAT
 541  CAAAAGCCCA ATTATACAGA AATCCGACAC TACTGCAATC ACTGGCGAAA TTTTGCTGAC
 601  ATTGATGATT CCTGGAAAAG TATAAAGAGT ATCTTGGACT GGACATCTTT TAACCAGGAG
 661  AGAATTGTTG ATGTTGCTGG ACCAGGGGGT TGGAATGACC CAGATATGTT AGTGATTGGC
 721  AACTTTGGCC TCAGCTGGAA TCAGCAAGTA ACTCAGATGG CCCTCTGGGC TATCATGGCT
 781  GCTCCTTTAT TCATGTCTAA TGACCTCCGA CACATCAGCC CTCAAGCCAA AGCTCTCCTT
 841  CAGGATAAGG ACGTAATTGC CATCAATCAG GACCCCTTGG GCAAGCAAGG GTACCAGCTT
 901  AGACAGGGAG ACAACTTTGA AGTGTGGGAA CGACCTCTCT CAGGCTTAGC CTGGGCTGTA
 961  GCTATGATAA ACCGGCAGGA GATTGGTGGA CCTCGGTCTT ATACCATCGC AGTTGCTTCC
1021  CTGGTAAAG  GAGTGGCCTG TAATCCTGCC TGCTTCATCA CACAGCTCCT CCCTGTGAAA
1081  AGGAAGCTAG GGTTCTATGA ATGGACTTCA AGGTTAAGAA GTCACATAAA TCCCACAGGC
1141  ACTGTTTTGC TTCAGCTAGA AATACAATG  CAGATGTCAT TAAAGACTT  ACTTTAA
```

## FIG. 13